Biochemical and Molecular Studies of the Prion Protein

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Nathalie Braun

von

Kreuzlingen TG

Begutachtet von

Prof. Dr. Adriano Aguzzi

Prof. Dr. Burkhard Becher

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SUMMARY

The physiological role of the cellular prion protein (PrP^C) remains enigmatic. Mice deficient for PrP^C (*Prnp*$^{o/o}$) develop normally but are resistant to prion disease. However, elucidation of the physiological function of PrP^C is of highest importance and may be crucial for understanding prion diseases. Expression of amino-terminally truncated PrP^C (ΔPrP) or of its structural homologue Doppel (Dpl) in *Prnp*$^{o/o}$ mice causes cerebellar neurodegeneration and white matter spongiosis. The structural similarity between ΔPrP and Dpl and the similar phenotype observed in mice expressing these proteins indicate that they trigger neurotoxicity by shared mechanisms. By introducing a single allele of *Prnp*, Dpl as well as ΔPrP-induced neurotoxicity was abrogated, suggesting that ΔPrP and Dpl interfere with a cellular pathway normally controlled by full-length PrP.

In the first part of this thesis, we have investigated the mechanisms elicited by overexpression of ΔPrP and Dpl in mice leading to white matter pathology and cerebellar neurodegeneration. White matter disease consists of myelin degeneration and axonal pathology, which are repressed by selective expression of PrP^C in oligodendrocytes, suggesting a role for PrP^C in axon-myelin interaction and myelin maintenance. Consistent with this, we found that PrP^C and ΔPrP are present in myelin and that, by immunoprecipitating myelin lysates, several myelin proteins specifically interact with the full-length as well as the mutant prion protein. MAG, one of the PrP-binding myelin proteins, represents the most promising interactor, since it is a transmembrane protein that could link the GPI-anchored protein PrP and the cytoplasm to transduce the signal elicited by the binding of a ligand to PrP. The rescue of the white matter disease by PrP^C expression on oligodendrocytes is also compatible with the interaction between PrP^C and an axonal molecule. To test this hypothesis, ΔPrP^o mice were crossed with $p75^{NTR}$ deficient mice, since prion protein fragment (106-126) has been shown to interact with $p75^{NTR}$. However, no rescue of the pathology in ΔPrP^o mice was observed. Therefore, $p75^{NTR}$ does not seem to be implicated in ΔPrP induced pathologies.

In order to study the molecular mechanisms leading to neurodegeneration induced by ΔPrP and Dpl, we established an *in vitro* model using primary cerebellar granule cell (CGC) cultures from *Prnp*$^{o/o}$ mice transduced by lentivirus containing the sequences

of ΔPrP, Dpl and PrP. No cell death was induced by ΔPrP and Dpl compared to PrP, suggesting that expression levels may be insufficient, cell culture conditions may be inadequate or CGC death may depend on other cell types.

Prion diseases are characterized by accumulation of a misfolded isoform of the cellular prion protein (PrP^{Sc}). The precise role of misfolded, aggregated scrapie prion protein in the neurodegenerative process remains, however, controversial. Biochemical analysis of these aggregates is hampered by the difficulty of processing and analyzing proteins prone to aggregation.

In the second part of this thesis, we have developed a method to solubilize aggregated PrP^{Sc}, making them directly amenable to proteomic analysis by mass spectrometry. A new agent, the anionic detergent sodium 3-[(2-methyl-2-undecyl-1, 3-dioxolan-4-yl) methoxyl]-1-propane sulfonate (MPS), proved to be the most effective “solubilizer” of PrP^{Sc} aggregates. Moreover, the agent is compatible with ICAT technology, renders PrP^{Sc} PK-sensitive and reduces prion-infectivity titers by several orders of magnitude. The effective solubilization of PrP^{Sc} aggregates should allow the detection of PrP^{Sc} associated proteins, which might be important for conversion of PrP^{C} to PrP^{Sc}. Since mass spectrometry analysis enables femto- to attomole sensitivity and a high mass accuracy, this assay could provide the detection of low abundant PrP^{Sc} in easy accessible material such as urine and blood as well as absolute quantification of PrP^{Sc} by the use of ICAT labeling, representing new diagnostic tools of prion disease.

ZUSAMMENFASSUNG

Die physiologische Bedeutung der normalen, zellulären Form des Prion-Proteins (PrP^C) ist noch immer unklar. Mäuse, die kein PrP^C exprimieren (*Prnp*$^{o/o}$), entwickeln sich normal, sind aber resistent gegenüber der Infektion mit Prionen. Die Aufklärung der physiologischen Funktion von PrP^C ist jedoch von grösster Relevanz und könnte für unser Verständnis von Prionen-Erkrankungen entscheidend sein. Expression einer amino-terminal trunkierten Form von PrP^C (ΔPrP) oder des strukturellen Homologs Doppel (Dpl) in *Prnp*$^{o/o}$ Mäusen verursacht Neurodegeneration im Kleinhirn und Spongiose der weissen Hirnsubstanz. Die strukturelle Ähnlichkeit zwischen ΔPrP und Dpl sowie der gleichartige Phänotyp, der durch die Expression von ΔPrP und Dpl in Mäusen hervorgerufen wird, legen nahe, dass sie Neurotoxizität über einen vergleichbaren Mechanismus induzieren. Die Co-Expression eines *Prnp* Allels führt zur Aufhebung der Neurotoxizität von ΔPrP und Dpl, was bedeuten könnte, dass ΔPrP und Dpl mit einem zellulären Signalweg interferieren, der normalerweise durch PrP^C kontrolliert wird.

Im ersten Teil dieser Doktorarbeit wurden die Mechanismen untersucht, die durch Überexpression von ΔPrP und Dpl in Mäusen zu pathologischen Veränderungen im Bereich der weissen Substanz und zu zerebellärer Neurodegeneration führen. Die Pathologie der weissen Substanz besteht aus einer Degeneration des Myelins sowie der Axone, welche durch selektive Expression von PrP^C in Oligodendrozyten unterdrückt wird, was eine Funktion von PrP^C in der Interaktion zwischen Axon und Myelin und bei der Myelinerhaltung nahe legt. Damit im Einklang haben wir gefunden, dass PrP^C und ΔPrP im Myelin vorkommen und dass, bei Immunopräzipitationen von Myelin, vier Myelin Proteine spezifisch mit dem zellulären und dem mutanten Prion-Protein interagieren. MAG, eines der PrP-bindenden Myelin-Proteine, ist wohl der vielversprechendste Bindungspartner, weil es ein transmembranäres Protein ist, das als Verbindung zwischen dem GPI-verankertem Prion-Protein und dem Zytoplasma fungieren und ein Signal weiterleiten könnte, welches durch Bindung eines Liganden an PrP^C ausgelöst wird. Die Unterdrückung der Leukoenzephalopathie durch die Expression von PrP^C in Oligodendrozyten könnte sich auch durch eine Interaktion zwischen PrP^C und einem axonalen Protein ereignen.

Wir haben diese Hypothese getestet, indem wir ΔPrP^{o} Mäuse mit p75^{NTR} defizienten (p75$^{o/o}$) Mäuse gekreuzt haben. Es konnte gezeigt werden, dass das Prion-Protein Fragment (106-126) mit p75^{NTR} interagiert. Die Pathologie der ΔPrP^{o} Mäuse liess sich jedoch durch die Kreuzung mit p75$^{o/o}$ Mäusen nicht beseitigen. Folglich scheint p75^{NTR} für die ΔPrP-induzierten Pathologien nicht von Bedeutung zu sein.
Um die molekularen Mechanismen, die zur zerebellären Neurodegeneration führen, untersuchen zu können, haben wir ein *in vitro* Modell erstellt, in dem primäre zerebelläre Körnerzellen von *Prnp*$^{o/o}$ Mäusen mit Lentiviren, die PrP, ΔPrP und Dpl cDNAs enthalten, transduziert wurden. Es wurde jedoch kein Zelltod durch ΔPrP und Dpl verglichen mit PrP induziert, was ineffiziente Expressionslevel, inadäquate Kulturbedingungen oder Abhängigkeit der CGCs von anderen Zelltypen suggeriert.

Prionen-Erkrankungen sind durch Ablagerungen einer abnorm gefalteten Form des zellulären Prion-Proteins (PrP^{Sc}; PrP Scrapie) charakterisiert. Die genaue Rolle des abnorm gefalteten, aggregierten PrP^{Sc} im neurodegenerativen Prozess ist jedoch umstritten. Biochemische Untersuchungen dieser Aggregate sind durch die Schwierigkeit, aggregierende Proteine zu prozessieren und zu analysieren, behindert.
Im zweiten Teil dieser Doktorarbeit, haben wir deshalb eine Methode einwickelt, um PrP^{Sc} Aggregate zu solubilisieren und um sie einer Analyse mittels Massen Spektrometrie zugänglich zu machen. Ein neuer Wirkstoff, das anionische Detergens Sodium 3-[(2-methyl-2-undecyl-1, 3-dioxolan-4-yl) methoxyl]-1-Propansulfonat (MPS), erwies sich als effektivstes Lösungsmittel der PrP^{Sc} Aggregate. Ferner ist der Wirkstoff mit der ICAT Technologie kompatibel, macht PrP^{Sc} PK-sensitiv und reduziert den Prion-Infektiositäts-Titer um mehrere Grössenordnungen. Die effiziente Solubilisierung der PrP^{Sc} Aggregate sollte erlauben, PrP^{Sc} assoziierte Proteine zu detektieren, welche für die Konversion von PrP^{C} in PrP^{Sc} wichtig sein könnten. Da Massenspektrometrie femto- bis attomole Sensitivität und grosse Massengenauigkeit erlaubt, wird diese Methode vielleicht die Entdeckung geringer Mengen von PrP^{Sc} in Urin und Blut sowie die absolute Quantifizierung von PrP^{Sc} mittels ICAT Technologie ermöglichen, was neue Tests für Prionen-Erkrankungen bedeutet.

DEFINITIONS

Adapted from Aguzzi and Weissmann (Aguzzi and Weissmann, 1997)

Prion: from ***pr***oteinaceous ***i***nfectious ***on***ly. Agent of transmissible spongiform encephalopathy (TSE), with unconventional properties. The term does not have structural implications other than that a protein is an essential component.

'Protein-only' hypothesis: Maintains that the prion is devoid of informational nucleic acid, and that the essential pathogenic component is protein (or glycoprotein). Genetic evidence indicates that the protein is an abnormal form of PrP (PrP* or, perhaps, PrP^{Sc}). The association with other 'non-informational' molecules (such as lipids or glycosamino glycans) is not excluded.

PrP^{C} or PrP-sen: The naturally occurring form of the mature *Prnp* gene product. Its presence in a given cell type is necessary, but not sufficient, for replication of the prion.

PrP^{Sc} or PrP-res: An 'abnormal' form of the mature *Prnp* gene product found in tissue of TSE sufferers, defined as being partly resistant to digestion by proteinase K under standardized conditions. It is believed to differ from PrP^{C} only (or mainly) conformationally, and is often considered to be the transmissible agent or prion (however, see PrP*).

PrP*: Within the framework of the protein-only hypothesis, PrP* is defined as the form of PrP constituting the essential (or only) component of the prion. It may, but need not, be identical to PrP^{Sc}.

GENERAL INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies

Prion diseases (PDs) or transmissible spongiform encephalopathies (TSEs) are rare fatal neurodegenerative disorders of humans and animals, encompassing scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob disease (CJD) in humans (Aguzzi et al., 2001). PDs may present as genetic, infectious, or sporadic disorders, all of which involve modification of the prion protein (PrP).

They are usually characterized by neuronal loss and spongiform degeneration of the brain accompanied by the appearance of activated astrocytes and microglia. Most distinctive, however, is the accumulation in the central nervous system of the host-encoded prion protein that is designated PrP^{Sc}, an abnormally folded form of the normal cellular prion protein (PrP^{C}). PrP^{Sc} can demonstrate properties of classic amyloid and form β-sheet rich, insoluble protein deposits that exhibit birefringence after staining with the amyloid dye Congo red.

Therefore, TSEs are often considered another type of amyloid disease such as Alzheimer's or Huntington's disease. However, prion diseases are unique among amyloid diseases in that they are transmissible. Homogenization of brain tissue from affected individuals and intracerebral inoculation into another individual of the same species will typically reproduce the disease. This important fact was recognized more than half a century ago in the case of scrapie (Cuille and Chelle, 1939), a prion disease that affects sheep and goats.

Much evidence suggests that the abnormal form of PrP may be critical in the transmission and pathogenesis of TSEs. Indeed, it has been proposed, but not yet proven, that an abnormal PrP is the infectious TSE agent or prion (Prusiner, 1982). Prions are defined as proteinaceous infectious particle that are devoid of nucleic acid and seem to be composed exclusively of PrP^{Sc}.

Prion diseases in humans

Several types of neurodegenerative syndromes have been recognized as prion diseases in humans: Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), Kuru, fatal familial insomnia (FFI), and fatal sporadic insomnia (FSI). They can be subdivided into three groups - acquired, sporadic, and inherited forms. Acquired conditions include diseases transmitted by dietary infections, such as Kuru or by other infection routes like iatrogenic contact with infected material during brain surgery. Sporadic CJD accounts for the majority of (about 85%) human prion disease at an incidence of about 0·4–1·8 cases per 1 million people per year world-wide, with an equal incidence in men and women (Brandel et al., 2000). The etiology of sporadic CJD is unknown, although hypotheses include somatic PRNP mutation, or the spontaneous conversion of PrP^{C} into the disease associated PrP^{Sc} as a rare event. Homozygosity at a common coding polymorphism at codon 129 of PRNP encoding either methionine or valine predisposes to the development of sporadic and acquired CJD (Palmer et al., 1991).

About 15% of human prion diseases are associated with autosomal dominant pathogenic mutations in PRNP (Doh-ura et al., 1989; Hsiao et al., 1989). These diseases include familial CJD, GSS and FFI. How pathogenic mutations in PRNP cause prion disease has yet to be resolved; however, in most cases, the mutation is thought to lead to an increased tendency of PrP^{C} to form PrP^{Sc}.

The emergence of a variant form of CJD (vCJD) in the United Kingdom in 1996 has been causally and experimentally linked to the UK BSE epidemic in the 1980s and early 1990s. The finding that BSE is transmissible to different animal species, unlike previously characterized prion diseases such as sheep scrapie, has raised enormous public health concerns worldwide. Although it is not yet possible to gauge the size of a potential vCJD epidemic, preliminary data indicate a significant dietary exposure to BSE-infected material in Britain and wider implications of the transmissibility of prion diseases.

Prion diseases typically exhibit a very long latency period between the time of infection and the clinical manifestation: this is the reason why these diseases were originally thought to be caused by “slow viruses”. From the viewpoint of interventional approaches, this peculiarity may be exploitable, since it opens a

possible window of intervention after infection has occurred, but before brain damage is being initiated. Prions spend much of this latency time executing neuroinvasion. During this process, little or no damage occurs to brain, and one might hope that its interruption may prevent neurodegeneration.

Prion diseases in animals

Animals TSEs include sheep and goat scrapie, the prototypic animal TSE that has been recognized more than 200 years ago, but also transmissible mink encephalopathy (Marsh and Hadlow, 1992), chronic wasting disease of mule deer and elk (Williams and Young, 1980) and bovine spongiform encephalopathy (BSE). Scrapie has been recognized as a disease in sheep for over two centuries, and was the first TSE to be shown as experimentally transmissible (Cuille and Chelle, 1939). Sheep and goats are susceptible to natural scrapie, and the disease occurs primarily in sheep of breeding age. Although the first cases have been described since the 18th century, its potential routes for natural infection remain elusive. In Great Britain, a previously unrecognized neurological disease in cattle was first defined in 1986: the pathologic changes in brains were similar to those characteristic of TSEs. This new form of TSE in cows, bovine spongiform encephalopathy (BSE) or mad-cow disease, rapidly developed into a major epidemic, primarily in the UK but also in other European countries. The emergence of BSE was likely to be caused by transmission of sheep scrapie to cattle, through infected feed prepared from rendered carcasses(Wilesmith et al., 1988). Chronic wasting disease (CWD) is a prion disease of captive and free-ranging mule deer and elk. Its spread appears to be enhanced by the abnormal population densities found in domestic facilities, although the actual mechanism of transmission is unknown. First recognized in captive animals, CWD has now also been detected in certain wild deer populations and wild elk. The fact that CWD is found in wild ruminants on the same range as cattle and sheep raises concern over the possibility that CWD could be transmitted to domestic animals and possibly might also pose a risk for human infection similar to BSE.

Neuroinvasion

Although the infectious agent is most efficiently propagated through intracerebral inoculation, peripheral infection is the natural route of transmission in most prion diseases. Oral administration is most probably involved in Kuru, BSE and the new variant CJD, while parenteral administration of growth hormone and gonadotropins has resulted in iatrogenic CJD. One of the perplexing features of prion diseases is the long incubation time from peripheral uptake of prions until the onset of clinical symptoms. The incubation time can be, at least in part, attributed to the slow kinetics by which prions reach their target organ, the central nervous system (CNS). The process by which prions gain access to the CNS is generally referred to as neuroinvasion (Aguzzi et al., 2001; Nicotera, 2001), which consists of two distinct phases. The first phase involves widespread colonization of lymphoreticular organs that is achieved by mechanisms that depend on M-cells (Heppner et al., 2001), macrophages (Prinz et al., 2002), B-lymphocytes (Klein et al., 1997; Klein et al., 1998), follicular dendritic cells (Montrasio et al., 2000) and complement factors (Klein et al., 2001). The second phase of neuroinvasion comprises transport of infectious prions on or in peripheral nerves, probably belonging to the autonomic nervous system, in a PrP^{C}-dependent fashion (Baldauf et al., 1997; Glatzel and Aguzzi, 2000). The innervation pattern of lymphoid organs is mainly sympathetic. Sympathectomy delays the transport of prions from lymphatic organs to the thoracic spinal cord, which is the entry site of sympathetic nerves to the central nervous system (Glatzel et al., 2001). Accordingly, the relative distance between FDCs and splenic nerves controls the velocity of prion neuroinvasion (Prinz et al., 2003). Direct invasion of the central nervous system by nerves might occur after peripheral infection (Race et al., 2000).

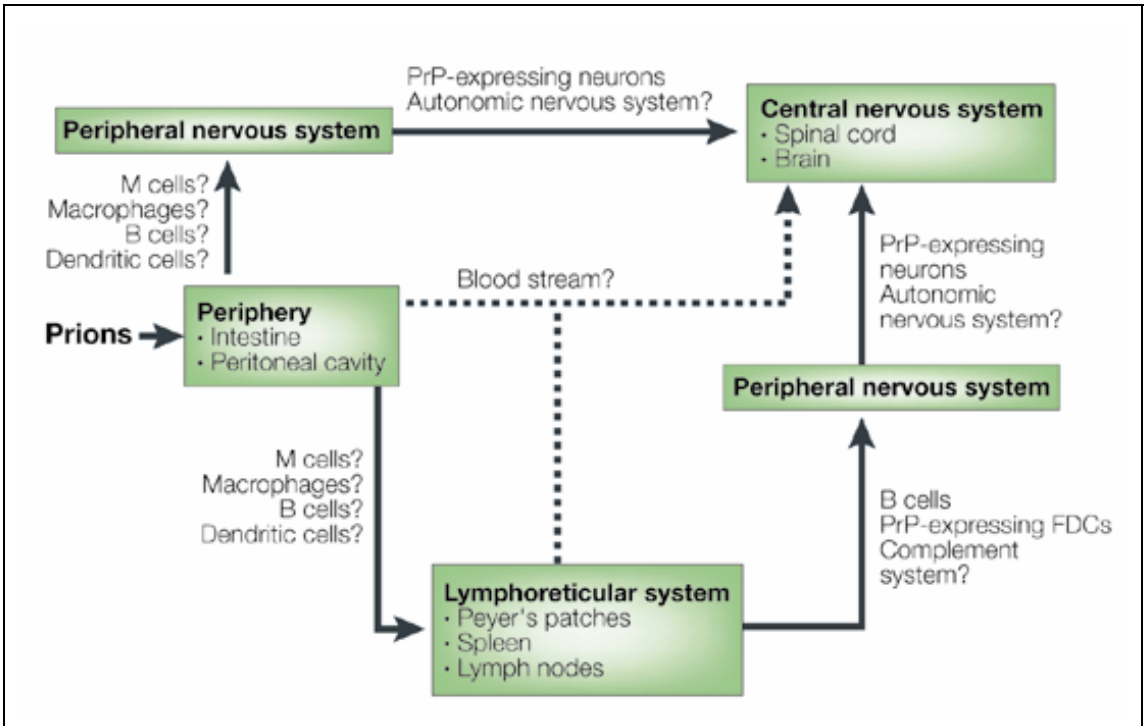


Figure 1. Model of prion neuroinvasion in mice. After peripheral inoculation, prions colonize the lymphoreticular system. Possible elements that might contribute to the transport into the lymphoreticular system are M cells, macrophages, B cells and dendritic cells. Required elements of secondary lymphoid organs for prion replication are functional follicular dendritic cells (FDCs) and B cells. In the progress of the disease, prions find access to the central nervous system, probably through elements of the autonomic nervous system. Alternatively, direct invasion of the central nervous system by nerves might occur after peripheral infection. Central nervous system infection through the blood cannot be excluded (Aguzzi et al., 2001).

The pathogenesis of prions

The mechanism of neurodegeneration in prion diseases is still poorly understood, and not much is known about the mechanism by which prions actually impair neuronal function and cause cell death. PrP^{Sc} accumulation in the brain is the hallmark of prion diseases, and PrP^{Sc} seems to be a major component of the infectious agent. But is PrP^{Sc} also directly responsible for the devastating CNS pathology typical of prion diseases? The essential role of PrP^{C} itself in prion propagation was confirmed when *Prnp* knock out mice were intracerebrally challenged with prions (Weissmann et al., 1993). They not only lacked any clinical signs of scrapie disease but also showed unaffected brains, which did not harbour any infectivity or protease resistant PrP; thus, ablation of PrP^{C} abolished prion replication and propagation. At this point it could be argued that PrP knockout mice were not affected because ablation of the normal host PrP removed the substrate for further conversion into PrP^{Sc}. Hence, the role of PrP^{Sc}-mediated neurotoxicity had to be addressed differently. To achieve a

sustained delivery of prions to the “prion-replication defective” brains, neurografts were introduced expressing high PrP levels and inoculated them with prions (Brandner et al., 1996b). These neurografts not only replicated and accumulated PrP^{Sc} and prion infectivity, but also delivered substantial amounts of prions to the host brain, notably without eliciting any clinical disease or neuropathological sign of spongiform encephalopathy. Hence, PrP^{C} is an indispensable substrate to elicit disease in the brain, and deposition of prions alone does not support neurodegeneration. In addition, depletion of PrP^{C} from neurons of scrapie-infected mice reversed early spongiform change and prevented neuronal loss and progression of clinical disease (Mallucci et al., 2003). This occurred despite the accumulation of extraneuronal PrP^{Sc} to levels seen in terminally sick wild-type animals. Therefore, PrP^{Sc} aggregates are unlikely to fully account for prion pathology.

Protein-only prion hypothesis

The protein-only hypothesis was first outlined in general terms by Griffith (Griffith, 1967), and formulated in more detail by Stanley B. Prusiner (Prusiner, 1982). It states that the partially protease-resistant and detergent-insoluble PrP^{Sc} is identical with the infectious agent and that the infectious agent is devoid of nucleic acid. Additionally, the propagation of the agent is thought to be mediated by PrP^{Sc} and to require conformational change from PrP^{C} to PrP^{Sc} molecules. Although the exact physical nature of the infectious agent is still controversial, a wealth of evidence supports this hypothesis. The primary amino acid sequence of PrP^{C} is a main determinant of the species barrier of prions. Linkage to mutations in the PrP^{C} gene was shown for all familial forms of human prion diseases (Prusiner et al., 1990). Mice devoid of PrP^{C} ($Prnp^{0/0}$) are resistant to infection by prions, and PrP^{C} is required for prion spread in the central nervous system (Brandner et al., 1996b; Büeler et al., 1993; Büeler et al., 1992). Moreover, the fact that the infectious agent is largely resistant to heat and irradiation also supports the protein-only hypothesis and against a viral hypothesis (for review see (Taylor, 2000)). However, the ultimate proof for the protein-only hypothesis, the in vitro conversion of recombinant PrP^{C} into infectious PrP^{Sc}, has turned out to be challenging, even though the cell-free conversion from PrP^{C} to a protease-resistant isoform by incubating PrP^{C} with PrP^{Sc} can be achieved relatively efficiently (Hill et al., 1999; Horiuchi and Caughey, 1999b; Kocisko et al., 1994;

Saborio et al., 2001). Only lately, the group of Prusiner reported for the first time the conversion of a recombinant truncated mouse prion protein (89-231) (MoPrP (89-231)) into amyloid fibrils that were infectious. Transgenic mice expressing MoPrP (89-231) were inoculated with these fibrils. The mice developed neurological dysfunction and brain extracts showed protease-resistant PrP by Western blotting (Legname et al., 2004). However, protease-resistance does not always correlate to infectivity (Hill et al., 1999; Kocisko et al., 1994; Riesner et al., 1996) and vice versa. Moreover, protease-sensitive scrapie prion protein has been reported recently in heterogeneous aggregates (Safar et al., 2000; Safar et al., 1998; Tzaban et al., 2002) and PrP^{C} may undergo disease-associated, diagnostically important modifications that do not lead to protease resistance (protease-sensitive PrP^{Sc}, or $sPrP^{Sc}$).

Another important experiment to prove the protein-only hypothesis involves abolishing the structure (and infectivity) of the disease-associated prion protein with specific salts, and then attempting to restore infectivity by reforming the original structure. This has so far failed (Prusiner et al., 1993).

Conversion of PrP^{C} into PrP^{Sc}

It has been suggested that PrP^{Sc} induces conversion of PrP^{C} into PrP^{Sc} by conformational change. In order to explain the mechanism by which a misfolded form of PrP could induce the refolding of native, normal PrP molecules into the abnormal conformation, two distinct models have been postulated: (a) the template assistance or 'refolding' model, and (b) the nucleation-polymerization or 'seeding' model (Figure 2). The first model postulates an interaction between exogenously introduced PrP^{Sc} and endogenous PrP^{C}, which is induced to transform itself into further PrP^{Sc}. A high energy barrier may prevent spontaneous conversion of PrP^{C} and PrP^{Sc} at detectable rates (Gajdusek, 1988; Prusiner et al., 1990). This reaction may involve extensive unfolding and refolding of the protein to explain the postulated high energy barrier and could be dependent on an enzyme or chaperone, provisionally designated as protein X (Telling et al., 1995). In the nucleation model, PrP^{C} and PrP^{Sc} are in equilibrium strongly favoring PrP^{C} (Come et al., 1993; Jarrett and Lansbury, 1993; Lansbury and Caughey, 1995). Only if several monomeric PrP^{Sc} molecules are mounted into a highly ordered seed, further monomeric PrP^{Sc} can be recruited and eventually aggregates to amyloid. Within such a crystal-like seed, PrP^{Sc} becomes

stabilized. Fragmentation of PrP^{Sc} aggregates increases the number of nuclei, which can recruit further PrP^{Sc} and thus results in apparent replication of the agent. Consistent with the latter model, cell-free conversion studies indicate that PrP^{Sc} aggregates are able to convert PrP^{C} into a protease-resistant PrP isoform (Bessen et al., 1995; Bessen et al., 1997; Kocisko et al., 1996; Kocisko et al., 1995).

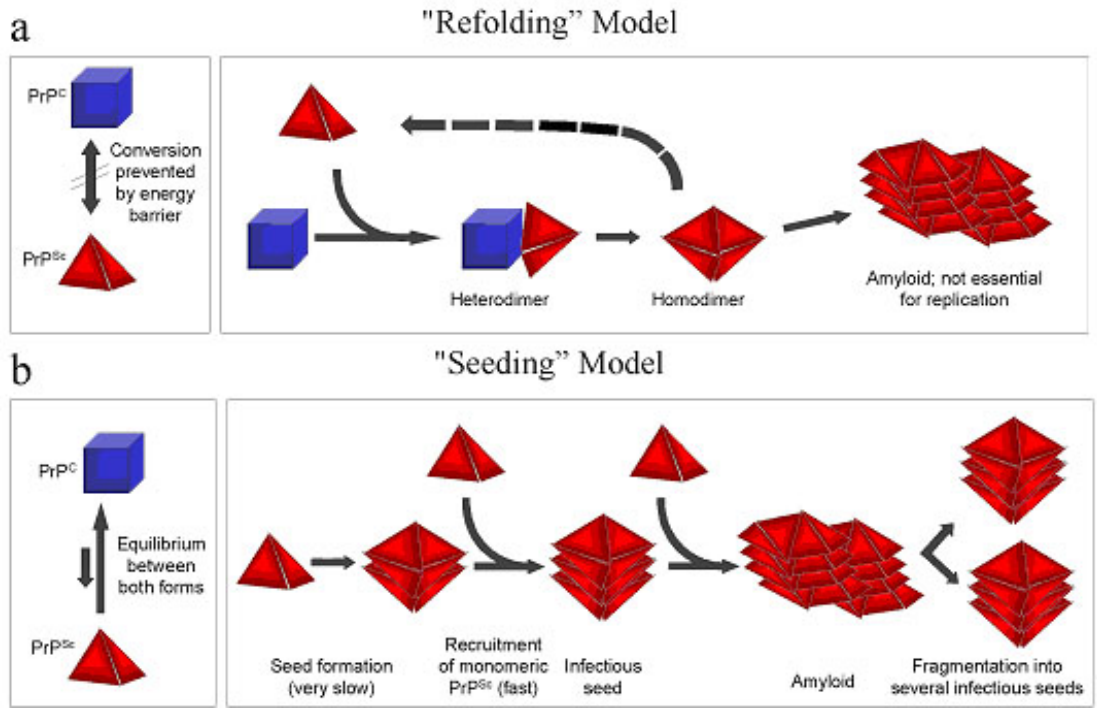


Figure 2. Models for the conformational conversion of PrP^{C} into PrP^{Sc}. A The 'refolding' or template assistance model **B** the 'seeding' or nucleation-polymerization model (Aguzzi and Heppner, 2000).

The prion protein

Cellular form of the prion protein (PrP^C) and its disease-associated isoform (PrP^{Sc})

The cellular form of the prion protein (PrP^C) consists of 254 amino acids. It is a conserved sialoglycoprotein of mainly unknown function, which is usually anchored to membranes via glycosyl-phosphatidylinositol (GPI). Nuclear magnetic resonance studies have evidenced the structure of the prion protein (Fig.1) (James et al., 1997; Riek et al., 1996; Riek et al., 1997; Zahn et al., 2000). It comprises three α-helices (amino acids 144-154, 175-193 and 200-219) and a small antiparallel ß-sheet (amino acids 128–131 and 161–164). The C-terminal part (*i.e*. amino acids 126–231) contains the complete globular part of the structure, whereas the N-terminus (*i.e*. amino acids 23–125) was flexible. Close to the small ß-sheet, a disulphide bridge connects helix 2 and helix 3.

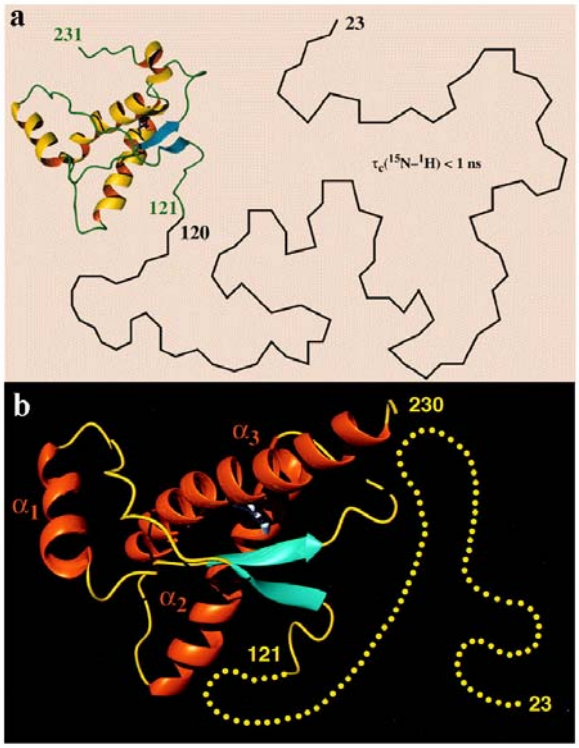


Figure 3. A The three-dimensional structure of a recombinant murine PrP^C expressed in *Escherichia coli*. The amino-terminal polypeptide segment 23-120 is flexibly disordered (reprinted from (Korth et al., 1997) **B** Cartoon of the three-dimensional structure of the intact human prion protein, hPrP (23-230). The helices are red, the β-strands cyan, the segments with non regular secondary structure within the C-terminal domain yellow, and the flexibly disordered "tail" of residues 23-121 is represented by yellow dots (Zahn et al., 2000).

In addition, PrP^{C} contains two N-linked complex-type oligosaccharides at positions 181 and 197. Accordingly, western blot analysis of PrP reveals three major bands, reflecting PrP that has two, one, or no glycosylation sites occupied. Glycosylation is important as a signal for correct intracellular trafficking of PrP^{C} and possibly also for selective targeting of PrP^{Sc} to specific brain regions (DeArmond et al., 1999; DeArmond et al., 1997). The amino acid sequence of PrP^{Sc} is identical to the one of PrP^{C}. PrP^{C} is converted into PrP^{Sc} through a process that changes its conformation. First indications for clear differences in the secondary structure came from spectroscopic measurements applying circular dichroism and infrared spectroscopy (Caughey et al., 1991; Safar et al., 1993). From those measurements, α-helix and ß-sheet contents could be determined. They show clearly that PrP^{C} is dominated by α-helices and has only little ß-sheet content, whereas PrP^{Sc} is characterized by similar amounts of α-helices and ß-sheets. This structural transition is accompanied by profound changes in the physicochemical properties of the prion protein. While PrP^{C} is soluble in mild detergents and sensitive to proteinase K (PK) digestion, PrP^{Sc} forms insoluble aggregates and is partially resistant to PK (Oesch et al., 1985); Meyer, 1986 #159}. The insolubility of PrP^{Sc} impedes its structural analysis by NMR. The PK resistance of PrP^{Sc} produces an N-terminally truncated form of PrP^{Sc}, designated $PrP^{27\text{-}30}$ according to its molecular weight on SDS-PAGE. The “full-length” PrP^{Sc} is cleaved around amino acid 90 and the cleavage product ($PrP^{27\text{-}30}$) is fully infectious (McKinley et al., 1991).

Expression pattern of PrP^{C}

The cellular prion protein is highly conserved in mammals and paralogues are present in turtle (Simonic et al., 2000), fish (Rivera-Milla et al., 2003; Suzuki et al., 2002) and amphibians (Strumbo et al., 2001), suggesting an important function of PrP^{C}. The gene encoding the cellular isoform of the prion protein is located on chromosome 20 in humans and on chromosome 2 in mice. It has been cloned by Charles Weissmann and termed *Prnp* in mouse and *PRNP* in humans (Basler et al., 1986). In all known mammalian and avian *Prnp* genes, a single open reading frame encodes PrP^{C}. The *Prnp* genes, of mice, sheep and cattle contain three exons, while those of hamsters and humans span over two exons (Lee et al., 1998). Within the genome, *Prnp* is present as

a single copy gene and does not show any homologies to other known cellular genes. *Prnp* mRNA is constitutively expressed in the adult organism and developmentally regulated during mouse embryogenesis (Manson et al., 1992; Miele et al., 2003). High levels of PrP^C are expressed in a broad range of tissue and cells including heart, skeletal muscle and kidney (Ford et al., 2002b), but its highest expression levels are detected in the central nervous system (Ford et al., 2002a). PrP^C expression has been mostly associated with neurons, particularly with synaptic membranes, although it is also expressed by other neural cell lineages like astrocytes and oligodendrocytes (Moser et al., 1995).

Biosynthesis of PrP^C

Like other membrane proteins, PrP^C is synthesized in the rough endoplasmic reticulum (ER) and transits the Golgi on its way to the cell surface. During its biosynthesis, the N-terminal hydrophobic signal sequence of 22 amino acids guides PrP to the membrane of the ER, where it is cleaved off. After translocation, 23 amino acids are removed from the C-terminus and a glycosylinositol-phospholipid (GPI) anchor is added (Stahl et al., 1987). In the ER, high mannose glycans are attached to the 25 kDa polypeptide at two asparagines residues and processed to hybrid or complex glycans during the passage through the Golgi apparatus (Bolton et al., 1985). The mature PrP^C is transported within secretory vesicles to the external cell surface to which it is anchored by the GPI-moiety. Recent work has shown that PrP^C undergoes two posttranslational cleavages as part of its normal metabolism. One cleavage occurs within the GPI anchor and releases the polypeptide chain into the extracellular medium (Borchelt et al., 1990). The second cleavage is proteolytic and occurs within a segment of 16 hydrophobic amino acids that is completely conserved in all cloned PrP species (Harris et al., 1993). The physiological significance of the cleavages remains speculative. The hypothesis was advanced that, if PrP^C functions as a cell surface receptor, the cleavages may represent mechanisms of receptor downregulation. PrP^C does not remain on the cell surface after its delivery there but, rather, constitutively cycles between the plasma membrane and an endocytic compartment (Caughey et al., 1989; Harris et al., 1996).

The physiological function of PrP^C

The physiological function of PrP^C remains elusive. Much hope was pinned on the use of PrP^C knock-out mice to unveil the function of the protein, but no obvious phenotype was observed (Büeler et al., 1992). Even postnatally induced *Prnp* ablation does not elicit any phenotype (Mallucci et al., 2002). Aging mice show demyelination in the peripheral nervous system, albeit without clinical symptoms (Nishida et al., 1999). A number of subtle abnormalities, e.g. abnormalities in synaptic physiology (Collinge et al., 1994) and in circadian rhythms and sleep (Tobler et al., 1996), have been described in PrP-deficient mice, but their molecular basis is undefined. The only definite phenotype of $Prnp^{0/0}$ mice is their resistance to prion inoculation (Büeler et al., 1993).

Several physiological roles for PrP^C have been proposed, in particular cell adhesion, signaling, neuroprotection and metabolic functions related to its copper-binding properties.

Cell culture experiments revealed a constitutive internalization process of PrP^C from the plasma membrane into endocytic organelles, yet most of the protein recycles back to the membrane without degradation (Shyng et al., 1993). The existence of a recycling pathway suggests that one physiological role of PrP^C might be to serve as a receptor for uptake of an extracellular ligand. One attractive candidate for such a ligand is the copper ion. It has been shown that copper ions at physiologically relevant concentrations rapidly and reversibly stimulate endocytosis of PrP^C from the cell surface (Pauly and Harris, 1998). Moreover, it has been reported that PrP^C can bind copper ions and possesses superoxide dismutase activity (Brown and Besinger, 1998; Brown et al., 1997). Additionally, amino-proximal truncated PrP^C seemed to depress endogenous dismutase activity (Sakudo et al., 2003), suggesting a role for copper binding of the N-terminal octapeptide-repeat segment. However, PrP^C does not make any measurable contribution to dismutase activity *in vivo* (Hutter et al., 2003; Waggoner et al., 2000).

Given the localization of PrP as a GPI-anchored protein in rafts or caveolae, it has been proposed that PrP, as with other GPI-anchored proteins, could be involved in signal transduction (Jacobson and Dietrich, 1999). Crosslinking of PrP^C with $F(ab)'_2$ antibody fragments has been reported to activate Fyn tyrosine kinase (Mouillet-Richard et al., 2000). Since Fyn is associated with cellular proliferation and cellular

survival, cell surface PrP might modulate neuronal survival. However, it was reported that PrP-mediated Fyn activation in cerebellar granule neurons might be responsible for neurite outgrowth rather than neuronal survival (Chen et al., 2003).

Other studies indicate that PrP^C may interact with components of signal transduction pathways, such as Grb2 (Spielhaupter and Schatzl, 2001). Grb2 is an adaptor protein that mediates growth factor receptor signals and also plays an important role in neuronal survival. Engagement of PrP^C with certain antibodies, a PrP^C binding peptide (Martins et al., 1997) or with a molecule called stress-inducible protein I (STI 1), leads to activation of both the cAMP/ protein kinase A and the Erk signaling pathways (Chiarini et al., 2002; Zanata et al., 2002). PrP^C-mediated activation of the cAMP/ protein kinase A (PKA) pathways have a cytoprotective effect in nervous tissue (Chiarini et al., 2002). Taken together, these studies clearly support a neuroprotective role for PrP through signal transduction events.

PrP has been shown to interact with the 37 kDa/67 kDa laminin receptor precursor (Gauczynski et al., 2001; Hundt et al., 2001; Rieger et al., 1997) and heparan sulfate (Warner et al., 2002), supporting a possible role in cell adhesion and/or signaling.

Maybe PrP^C does not possess any intrinsic biological activity, yet it modifies the function of other proteins. Multiple PrP^C interacting partners have been identified in the recent years: the antiapoptotic protein Bcl-2 (Kurschner and Morgan, 1995), caveolin (Gorodinsky and Harris, 1995; Harmey et al., 1995), N-CAM (Schmitt-Ulms et al., 2001) and neurotrophin p75 receptor (Della-Bianca et al., 2001). None of these interactors, however, have yet revealed a functional pathway in which PrP^C would be involved in vivo.

PART I

INTRODUCTION

Amino-terminal truncated PrP

Mice devoid of PrP develop normally but are resistant to scrapie (Büeler et al., 1993; Büeler et al., 1992). Introduction of a PrP transgene restores susceptibility to the disease. To identify the region of PrP necessary for this activity, PrP^C with amino-proximal deletions were introduced in PrP knockout mice. Ablation of the N-terminus up to amino acid 106 (PrPΔ21-106) was not deleterious and restored prion propagation in *Prnp*$^{0/0}$ mice (Flechsig et al., 2000; Shmerling et al., 1998). However, PrP lacking amino acid residues 32-121 and 32-134 (PrPΔ21-121 and PrPΔ21-134) caused early onset ataxia and impaired survival (Shmerling et al., 1998). Histological analysis of the brains of 2-3 months old animals showed massive pathological changes in the cerebellum: cerebellar granule cell degeneration, intense astrocytosis and irregularly distributed large vacuoles in the white matter in the pons. Expression of N-terminally truncated PrP targeted to Purkinje cells also leads to Purkinje cell loss and ataxia (Flechsig et al., 2003), suggesting that the promoter used in the ΔPrP mice is not active in Purkinje cells (Chiesa et al., 1998; Fischer et al., 1996). Reintroduction of one intact PrP^C allele leading to co-expression of a full-length prion protein in both models prevented this phenotype. This led to the concept that PrP^C and a functional homologue (π) were competing for a ligand or a receptor to transduce an important signal and that this homologue could take over PrP^C function in the absence of PrP^C explaining the phenotypic paucity in PrP deficient mice. The truncated mutant, having presumably the same affinity for the ligand as wild-type PrP, could compete away π in a dominant negative fashion but lack functionally competent domains to transduce the relevant signal, which would result in neurodegeneration (Fig. 4).

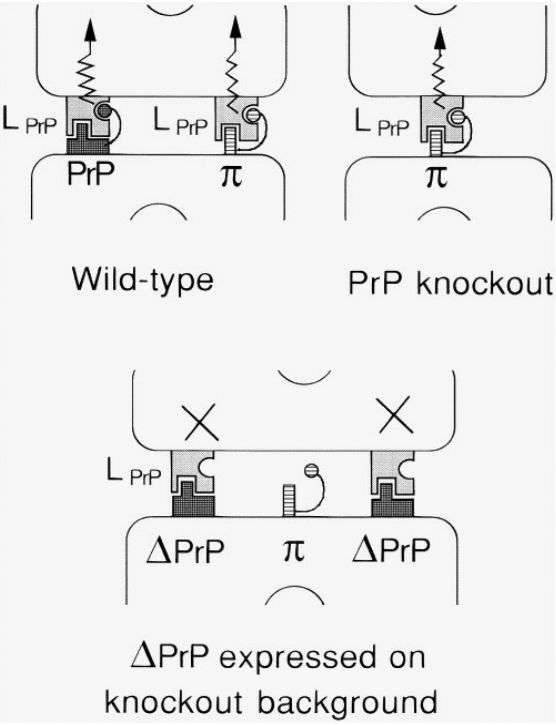


Figure 4. Model to explain effects of truncated PrP. PrP is composed of a globular part (dark hatched polygon) and a flexible tail (string-and-ball) (Riek et al, 1997) and may interact separately with a presumed ligand, "L_{PrP}", via these two domains, thereby eliciting a signal. The same signal might be elicited by the interaction of L_{PrP} with π, a conjectural protein that has the functional properties of PrP (top, left) explaining why PrP knockout mice show no phenotype (top, right). The model postulates that PrP lacking the flexible tail can interact with L_{PrP} without giving rise to a signal and that it can compete efficiently with π, thus acting as a dominant inhibitor of the latter (bottom) (Shmerling et al., 1998).

The doppelgänger of PrP – the protein Doppel (Dpl)

The discovery of Dpl

Different knockout strategies for disrupting *Prnp* have been employed (Fig. 5). All mutant mouse lines lacked significant regions of the *Prnp* open reading frame (ORF) and did not express PrP^C, but showed strikingly different phenotypes. The so-called Zurich I $Prnp^{o/o}$ mice as well as the Edinburgh $Prnp^{o/o}$ have deletions of two thirds of the *Prnp* ORF (Büeler et al., 1992; Manson et al., 1994). These mice were clinically healthy, although displaying discrete neurophysiological changes and demyelination of peripheral nerves on aging (Büeler et al., 1992; Collinge et al., 1994; Manson et al., 1994; Tobler et al., 1996). However, three other PrP knockout lines, Nagasaki (Ngsk), RcmO and Zürich II (ZrchII) $Prnp^{o/o}$, displayed early normal development but late-onset ataxia accompanied by cerebellar neurodegeneration mainly due to loss of

cerebellar Purkinje cells. In contrast to the Zurich I and Edinburgh PrP knockout mice, in the Ngsk, Rcm0, and ZrchII lines, not only the ORF, but also parts of intron 2 and the non-coding flanking regions were disrupted (Li et al., 2000; Moore et al., 1999; Sakaguchi et al., 1996).

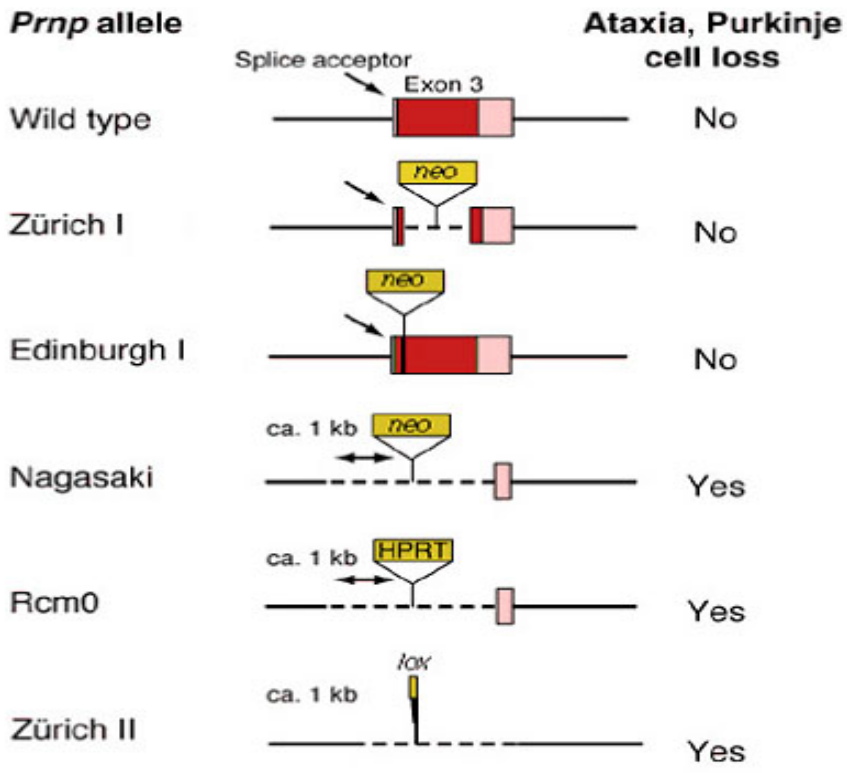


Figure 5. Various strategies used to disrupt the locus of the *Prnp* gene in the creation of different strains of *Prnp* knockout mice. The dotted line indicates the segment of *Prnp* DNA that has been deleted, and the green box indicates a sequence inserted into the gene. Dark red, ORF; pink, non-coding region; *neo*, neomycin phosphotransferase; HPRT, hypoxanthine phosphoribosyltransferase; *lox*, a 34-bp recombination site from phage P1 (Weissmann and Aguzzi, 1999).

Since introducing a PrP-transgene prevented the disease, this phenotype was first attributed to PrP (Nishida et al., 1999). However, by sequencing of a *Prnp*-containing cosmid, a novel gene 16 kb downstream of *Prnp* was discovered encoding a 173 residue protein (Moore et al., 1999). This protein was named Doppel (Dpl) for *d*ownstream of the *p*rion *p*rotein *l*ocus and the gene locus, *Prnd*. It then emerged that the gene targeting strategy in all ataxic PrP-deficient mice was associated with deletion of a splice acceptor site located on the coding exon of *Prnp* (Fig. 6). This modification effectively placed Dpl under transcriptional control of the *Prnp* promoter (Moore et al., 1999; Sakaguchi et al., 1996). While the *Prnp* promoter is strongly expressed in neuronal cells, the *Prnd* promoter is not. Therefore *Prnd* expression from

the *Prnp* promoter results in overproduction of Dpl in the brain, probably being responsible for neuronal degeneration. Further experiments have demonstrated an inverse correlation between the mRNA levels of *Prnd* and the onset of ataxia. Disease progression is accelerated by increasing *Prnd* levels, supporting the idea that ectopic Dpl expression, but not functional loss of PrP^{C}, might be responsible for neuronal degeneration in ataxic *Prnp*-deficient mice (Rossi et al., 2001). Moore and colleagues (Moore et al., 2001) reported that transgenic $Prnp^{o/o}$ mice expressing Dpl ubiquitously in brain develop severe ataxia associated with loss of both granule and Purkinje cells. The age of onset was inversely proportional to the level of Dpl expression, and 1-month-old mice were already affected. Introduction of a hamster PrP-encoding transgene resulted in complete abrogation of the cerebellar syndrome in animals expressing moderate levels, and partial abrogation in animals expressing high levels of Dpl.

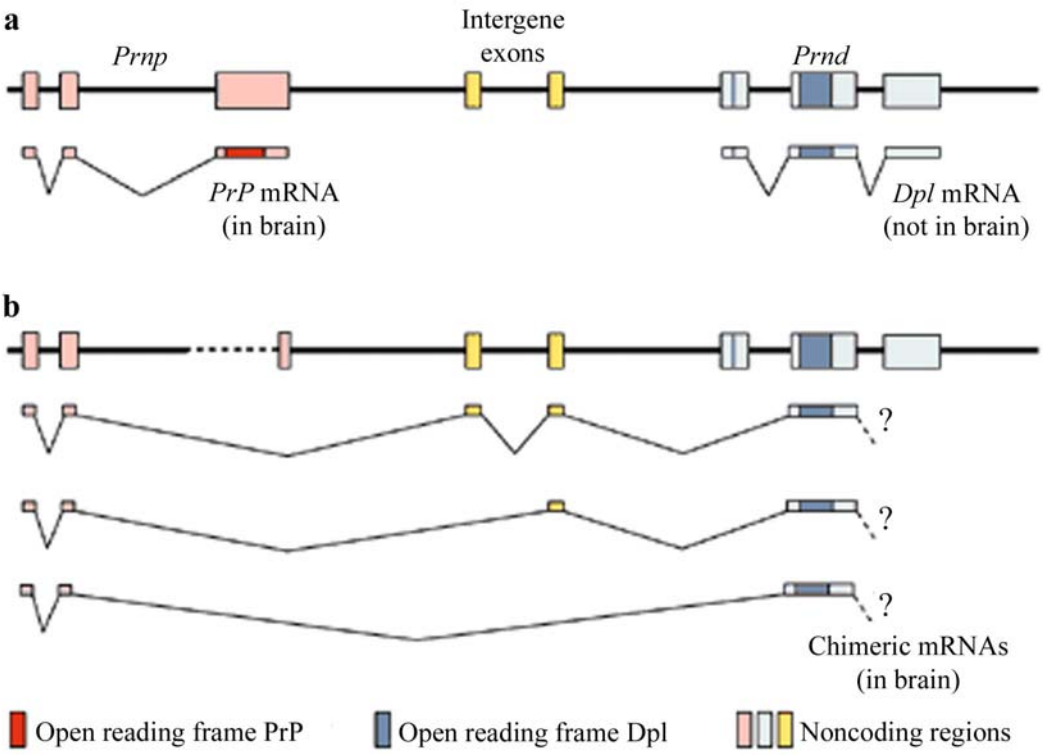


Figure 6. A prion protein's doppelgänger. The *Prnp* and *Prnd* loci and expression of the PrP and Dpl mRNAs and proteins that they encode. (a) Coding and non-coding exons of *Prnp*, *Prnd*, and intergenic exons of unknown function. (b) Deletion of the exon of *Prnp* containing an open reading frame and its flanking regions results in the formation of several chimeric mRNAs that comprise the first two exons of *Prnp* which are spliced directly or indirectly to the exon encoding Dpl (Weissmann and Aguzzi, 1999).

The structure of Dpl

Dpl is a GPI-anchored protein, expressed on the cell surface (Silverman et al., 2000). It displays 23% sequence identity with the C-terminal two thirds of PrP^C, but lacks the N-terminally located copper-binding octapeptide repeats and the highly conserved transmembrane region of PrP (Fig. 6). Contrary to the single disulfide bridge in PrP, Dpl has two disulfide bonds. The NMR structure of MoDpl (Mo et al., 2001) as well as HuDpl (Luhrs et al., 2003) has recently been resolved: both have an unstructured flexible tail at the N-terminus (residues 24-51) and a C-terminal globular domain comprising four α-helices and two short β-strand motifs showing a close similarity to MoPrP and HuPrP, respectively. Nonetheless, there are differences that may be functionally significant.

The phenotypic proximity and the structural similarity between Dpl and the truncated PrP (Fig. 7) suggest that they trigger neurotoxicity by shared mechanisms. By re-introducing a single allele of *Prnp*, Doppel as well as ΔPrP induced neurotoxicity were abrogated. The mechanism by which Dpl and ΔPrP cause cell death might interfere with a cellular pathway normally controlled by full-length PrP.

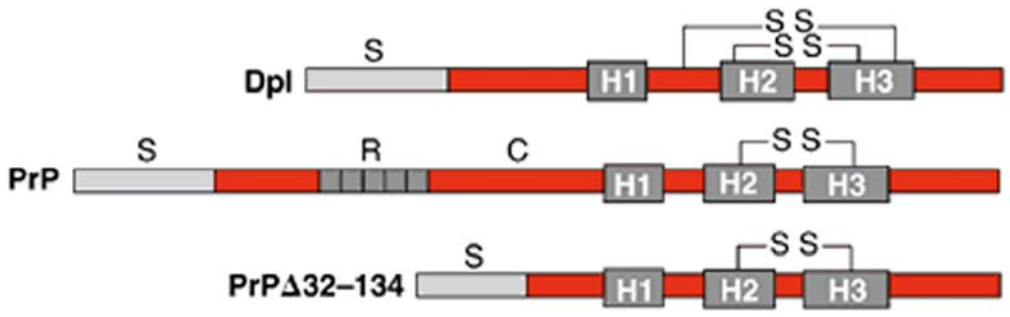


Figure 7. Comparison of predicted domains of Dpl with full-length PrP and with PrP in which amino acid residues 32 to 134 have been deleted (ΔPrP) (Weissmann and Aguzzi, 1999).

The expression of Dpl

The *Prnd* gene is evolutionarily conserved, suggesting an essential biological function (Aguzzi et al., 2001; Brown et al., 1999; Silverman et al., 2000). It is expressed in a more restricted manner than *Prnp*. By Northern and Western blot analysis, Dpl mRNA and protein are found in adult testis and heart, but are absent from the CNS in adult wild-type mice (Moore et al., 1999; Silverman et al., 2000). However,

significant amounts of *Prnd* mRNA transcripts have been detected during embryogenesis and in the brains of newborn mice, which argues for a possible function of Dpl in brain development (Li et al., 2000).

The physiological function of Dpl

To investigate the physiological function of Dpl, mice lacking Dpl were created. These mutants develop normally yet suffer from male infertility (Behrens et al., 2002). These mice show a significant number of malformed and less mobile spermatocytes suggesting a deficit in spermatogenesis. Only after partial disruption of the zona pellucida was *in vitro* fertilization of $Prnd^{o/o}$ partially restored, therefore implicating acromsome malformation as a crucial reason for sterility. These experiments revealed a clear function for Dpl in male reproductive system, however they provide no information about a possible agonistic or antagonizing functional relation with PrP^{C}. Therefore double *Prnp/Prnd* knockout mice were created (Genoud et al., 2004). Mice lacking both PrP^{C} and Dpl ($Prn^{o/o}$) develop normally but suffer from male infertility. The absence of compound pathological phenotypes in $Prn^{o/o}$ mice suggests the existence of alternative compensatory mechanisms. Alternatively, Dpl and PrP^{C} may exert distinct function despite having partly overlapping expression profiles.

The role of Dpl in prion pathogenesis was studied by grafting neural stems cells homozygous for the disruption of the *Prnd* locus in the brains of *Prnp* knockout mice. The *Prnd* null grafts were found to undergo normal neural differentiation, ruling out an important function of Dpl during brain development. After inoculation with scrapie, grafts lacking *Prnd* showed unimpaired prion pathogenesis and infectivity level similar to wild-type grafts (Behrens et al., 2001). Similarly, overexpression of Dpl in the brain of transgenic mice does not modulate prion pathogenesis (Tuzi et al., 2002). Despite the resemblance of Dpl to N-terminally truncated PrP^{C} protein that actually supports PrP^{Sc} propagation, Dpl protein seems not to be susceptible to conversion into "Dpl^{Sc}".

Outline of this work

Expression of amino-terminally truncated PrP^C (ΔPrP) or of its structural homologue Dpl causes cerebellar neurodegeneration that is prevented by co-expression of full-length PrP (Shmerling et al 1998; Rossi et al 2001). In addition to cerebellar neurodegeneration, ΔPrP expressing mice (ΔPrP^o mice) as well as Dpl expressing mice (*Prnp*$^{o/ZrchII}$ mice) suffer from white matter spongiosis. Electron microscopic analysis revealed myelin splitting and myelin degeneration associated with axonal degeneration of mainly large caliber axons, suggesting that ΔPrP and Dpl are myelinotoxic. By breeding mice that express PrP under the transcriptional control of the neuron specific enolase (NSE) promoter (termed NSE-PrP^o mice) and mice that express PrP under the transcriptional control of a fragment of the myelin basic protein (MBP) promoter (termed MBP-PrP^o mice) (Prinz et al 2004) with ΔPrP^o mice or *Prnp*$^{o/ZrchII}$ mice, it appeared that cerebellar neurodegeneration and white matter disease are two independent phenomena. ΔPrP^o; NSE-PrP mice and *Prnp*$^{o/ZrchII}$; NSE-PrP mice exhibit a partial rescue of the cerebellar neurodegeneration. However, white matter spongiosis was as severe as in ΔPrP^o mice. In the ΔPrP^o; MBP-PrP mice and *Prnp*$^{o/ZrchII}$; MBP-PrP mice exhibit the opposite phenotype: white matter spongiosis repression and severe and complete cerebellar neurodegeneration. Theses data underline the hypothesis that ΔPrP and Dpl act by the same mechanism to induce toxicity.

To elucidate the mechanism(s) by which ΔPrP and Dpl cause myelin pathology and neurodegeneration, we have chosen two different approaches: an in vivo model to study the processes leading to axon-myelin degeneration in ΔPrP mice in more detail, and an in vitro model to dissect the molecular mechanisms that lead to cerebellar granule cell death in mice expressing ΔPrP and Dpl.

PrP expression in oligodendrocytes specifically rescued the myelinopathy in ΔPrP^o mice. Therefore physiological oligodendroglial PrP^C expression may have an essential function in axon-glial interaction and in myelin maintenance. The mechanism leading to the rescue of axonal degeneration seems to be non-cell autonomous. ΔPrP and Dpl may interfere with this signaling pathway essential for axon-glial interaction and myelin maintenance and give rise to the observed leukoencephalopathy. We screened myelin lysates for interaction partners of PrP and ΔPrP. Two non-compact myelin

proteins, myelin associated glycoprotein (MAG) and 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP), and two compact myelin proteins, myelin basic protein (MBP) and myelin-associated oligodendrocytic basic protein (MOBP), bound specifically to both forms of the prion protein.

To gain insight into the mechanism of Dpl and ΔPrP induced neurotoxicity, we established an *in vitro* model that is amenable to genetic and biochemical analysis. Primary cell cultures of cerebellar granule cells (CGCs) isolated from PrP knock out mice were transduced with lentivirus expressing cDNAs of PrP^{C}, Dpl and ΔPrP. CGCs were successfully transduced with the different lentiviral constructs. However, we did not observe any cell death in cultured cerebellar granule cell death.

RESULTS

(A part of these results are submitted in Radovanovic I, Braun N et al, 2004)

Myelinopathy and axonal degeneration in ΔPrP^o mice

We found that, in addition to cerebellar granule cell (CGC) degeneration (Fig. 9a), mice expressing the amino-terminal truncated PrP (Δ32-134) suffer from widespread leukoencephalopathy (Fig. 9e). The widespread leukoencephalopathy in terminal sick ΔPrP^o mice (hemizygous for the PrPΔ32-134 transgene and devoid of both endogenous *Prnp* alleles) is characterized by coarse vacuolation of the white matter, encompassing the intrapontine part of cranial nerves, white matter of the cerebellum, the brain stem as well as the anterior and lateral spinal cord columns. On semi-thin sections, myelinated fibers in spinal cord and cerebellar white matter displayed severe axonal loss associated with large vacuoles, and degeneration of myelin sheath into condensed spheroids (Fig 9i). The abnormalities observed in ΔPrP^o mice are abolished when a wild-type *Prnp* transgene is introduced (Fig. 9d, h, l). White matter spongiosis is not associated with oligodendrocyte cell death or other neuronal cell death than the one of cerebellar granule cells (data not shown).

Electron microscopy analysis revealed axonal swelling containing organelles (Fig. 8 a and b). Other axons suffered from compromised adhesion to their myelin sheath, evident as periaxonal splitting over broad portions of the axolemma and detachment form the myelin wrap (Fig. 8c and d). Many axons had completely degenerated leaving behind empty myelin rings or collapsed and condensed residual myelin ghosts (Fig. 8e and f).

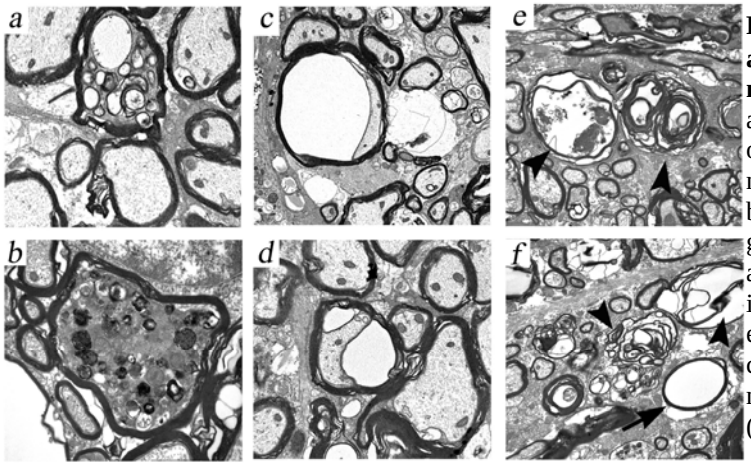


Figure 8. Electron microscopy analysis of spinal cord of ΔPrP^o mice. (a and b) axonal swelling and spheroids with accumulation of membranous organelles, mitochondria and undefined dense bodies, (c and d) defects in axon-glial adhesion, (e and f) more advanced stage of degeneration include axonal degeneration with empty myelin rings (arrow) and collapse of myelin in condensed multilamellar spheroid structures (arrowhead)

Rescue of myelinopathy and cerebellar granule cell degeneration

If myelin-producing oligodendrocytes were a primary target of mutant PrP^C, selective oligodendrocytic expression of PrP^C should repress white-matter pathology. ΔPrP^o were, therefore, bred to *tg*640 mice, which express full-length PrP^C under transcriptional control of the MBP promoter (referred to as MBP-PrP^o; Prinz et al 2004). Hemizygous expression of the MBP-PrP transgene in ΔPrP^o mice (referred to as MBP-PrP; ΔPrP^o) significantly delayed the onset of tremor and ataxia, whereas ΔPrP^o littermates displayed sever wasting and hind limb paralysis. Histological analysis showed repression but not complete rescue of axon-myelin degeneration and white matter vacuolation (Fig. 9f and j) and absolutely no rescue of CGCs was observed (Fig. 9b) in MBP-PrP; ΔPrP^o mice.

Alternatively, neuronal processes may conceivably represent the primary target of mutant PrP toxicity, with myelinopathy being secondary to axonal degeneration. To investigate this hypothesis, ΔPrP^o mice were crossed to mice expressing full-length PrP^C under transcriptional control of the neuron specific enolase (NSE) promoter (NSE-PrP^o mice). NSE-PrP^o mice express PrP^C in neurons, including CGCs, at higher levels than wild-type mice, and do not develop any spontaneous pathological phenotype (Giger O, Navaro B and Aguzzi A, unpublished data). ΔPrP^o; NSE-PrP mice displayed a delayed onset of tremor and ataxia. Histological analysis showed repressed cerebellar neurodegeneration (Fig 9c), but revealed leukoencephalopathy of similar severity as in ΔPrP^o mice (Fig 9g and k). Therefore, white matter disease and granule cell degeneration appear to be independent of each other. Co-expression of

either oligodendrocyte- or neuron-specific PrP^C restored long term survival (>500days) with stable or minimally progressing disease in both cases, whereas the mean survival time of ΔPrP^o littermates was around 100 days. However, only ubiquitous expression of PrP completely restored the pathology in ΔPrP^o mice (Fig. 9d, h and l).

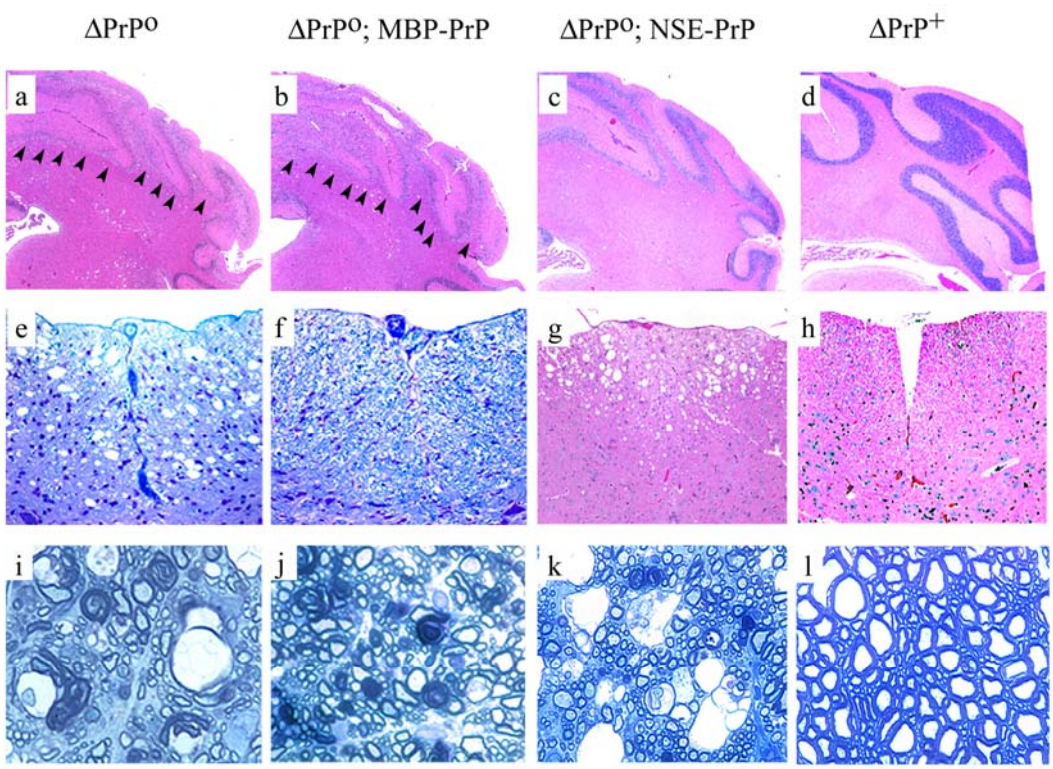


Figure 9. Myelinopathy and cerebellar granule cell degeneration are independent from each other. Cerebellar granule cell degeneration in ΔPrP^o mice (a). Neuron-specific expression of PrP rescue cerebellar granule cell degeneration (c), while oligodendrocyte specific expression does not (b). However, oligodendrocyte specific expression rescues myelinopathy (f), while neuron specific expression does not (g). Toluidine blue stained semi-thin sections through spinal cord show axonal loss with large vacuoles, and degeneration of myelin sheaths into condensed spheroids in ΔPrP^o mice and mice that express PrP^C selectively in neurons (i and k). ΔPrP^o; MBP-PrP mice show degenerated myelin spheroids but no vacuolation and an increased number of large caliber axons (j). Co-expression of PrP in ΔPrP^o mice completely restores the myelin anomalies as well as the cerebellar granule cell degeneration (d, h, and l).

No delay or abnormalities in myelination

Axon-myelin pathology may result from developmental myelination failure, or from degeneration of myelinated axon following normal development. This question was addressed by studying semi-thin spinal cord and cerebellar white matter sections from P10 and from P25 ΔPrP^+ and ΔPrP^o mice, to exclude any delay of initiation of

myelination or abnormality in myelination (Fig. 10). We have not observed any delay or abnormality in myelination based on toluidine blue semi-thin sections.

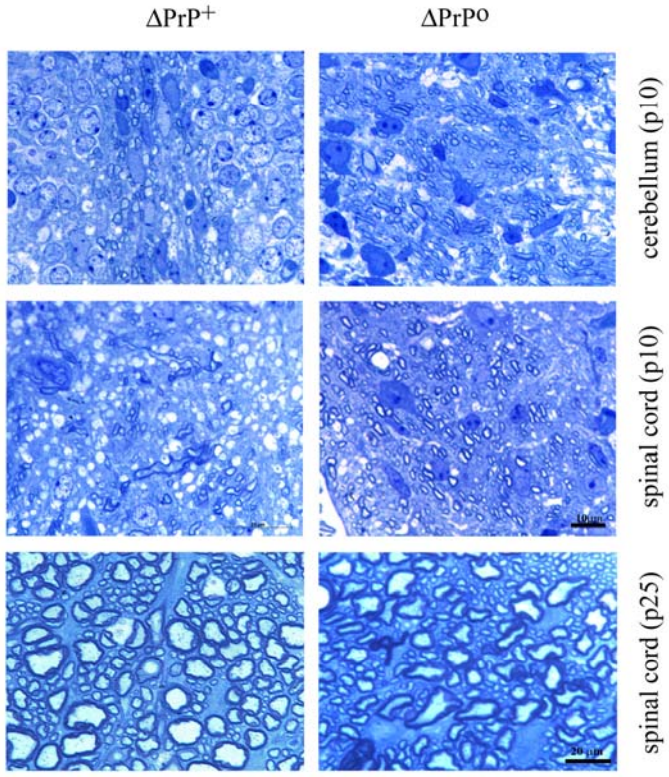


Figure 10. Neither delay nor abnormalities in myelination in spinal cord or cerebellum of p10 or p25 old ΔPrP^{o} mice. Magnification of cerebellum and spinal cord of p10 mice is 1000x, of spinal cord from p25 mice is 2000x.

PrP^{C} and ΔPrP are present in purified myelin

Oligodendrocyte-specific expression of PrP^{C} suppresses myelin-axon degeneration in ΔPrP^{o} mice. If PrP^{C} exerts a function in axon-glial interaction or myelin maintenance, it must be present in myelin.

Myelin was isolated from brains of *Prnp*$^{o/o}$, wild-type mice, MBP-PrP^{o}, NSE- PrP^{o} and ΔPrP^{o} mice (age: 2-3 months). Significant amounts of PrP^{C} were detected in myelin preparations of wt, ΔPrP^{o} and MBP-PrP mice (Fig 11Aa). However, we also found PrP^{C} in purified myelin of NSE-PrP mice (Figure 11Aa), whose oligodendrocytes do not express PrP^{C} (Fig 11Ba). Poor fractionation with neuronal or astrocytic components contaminating the myelin preparation appears to be unlikely, since essentially all GFAP and neurofilament proteins, which would be indicative of contamination, were removed (Fig. 11Ab and Ac). The presence of PrP^{C} in myelin of

NSE-PrP mice, therefore, suggests that axolemmal PrP undergoes high-affinity interactions with myelin membrane proteins, as shown for other proteins of the myelin-axolemmal complex (Menon et al 2003). Alternatively, neuronally synthesized PrP^C may be transferred onto myelin sheaths by "cell painting", which was shown to be highly efficient in the case of GPI-linked proteins (Anderson et al 1996). The strong, low molecular weight bands that can be observed for PrP of the MBP-PrP^o mice might be due to the degradation of the unglycosylated PrP, because glycosylation is known to influence the stability of proteins. The unglycosylated form seems to be predominant in myelinic PrP^C, similarly to what was observed in MBP-PrP^o mice (Prinz et al 2004).

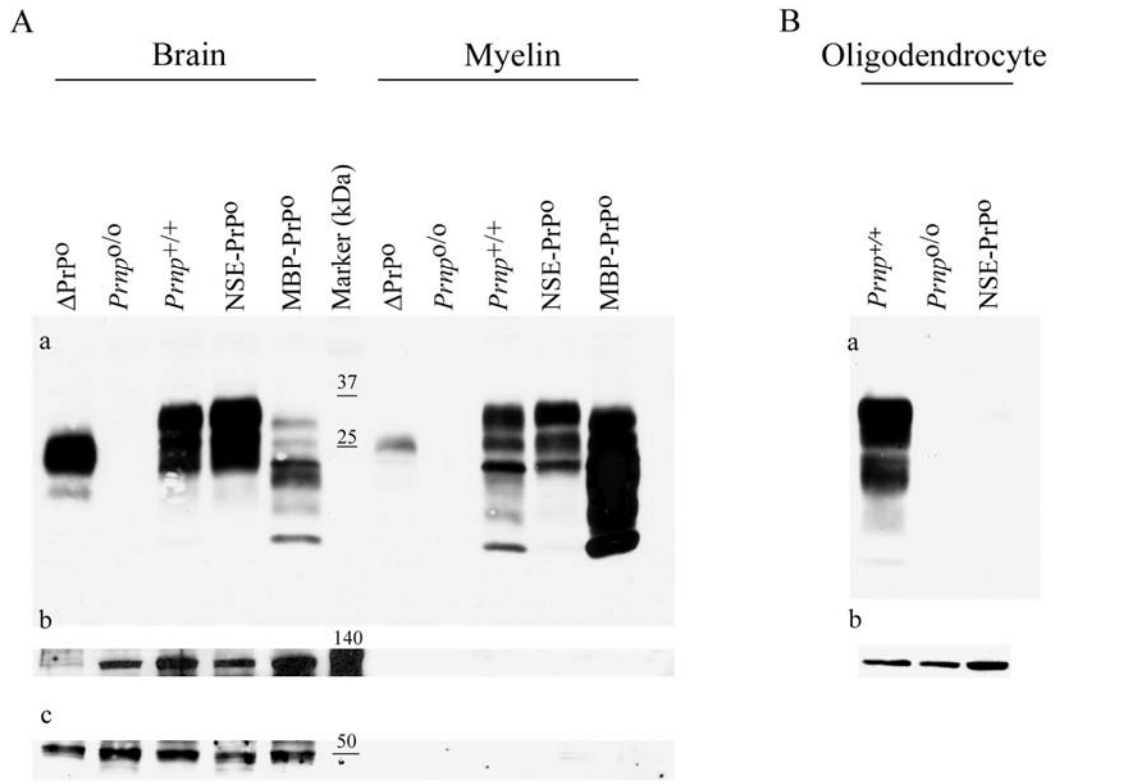


Figure 11. A (a) 10μg of brain homogenate and 5μg of myelin preparation of various genotypes are loaded in each lane. Myelin was tested for purity by GFAP (b), marker for astrocytes, and NF, marker for neuron (c). B (a) Equal amount of protein of the different oligodendrocyte lysates were loaded (b) actin loading control.

PrP^C is localized to the oligodendrocyte plasma membrane

PrP^C is a GPI-anchored protein that is processed through ER and Golgi and localizes into lipid rafts (Meier et al., 2003; Naslavsky et al., 1997). Since rafts are essential

features of all organellar membranes (Mayor and Riezman, 2004), other organellar membranes than myelin might co-purify during myelin isolation and could be responsible for the presence of PrP^C in myelin. Therefore, we analyzed the myelin preparations for the presence of Golgi components or ER components by specific markers. As figure 12a shows, no marker for either of them were present in the myelin membrane preparation. In addition, treatment of myelin membranes with PIPLC confirmed the presence of PrP in the myelin membrane. NCAM-120, another GPI anchored protein and major NCAM isoform in the myelin (Bartsch, 2003), is also released into the supernatant upon treatment with PIPLC. MAG, however, a transmembrane protein exclusively found in the myelin sheaths of both oligodendrocytes and Schwann cells, was not released into the supernatant, but instead remained in the pellet (Fig.12b).

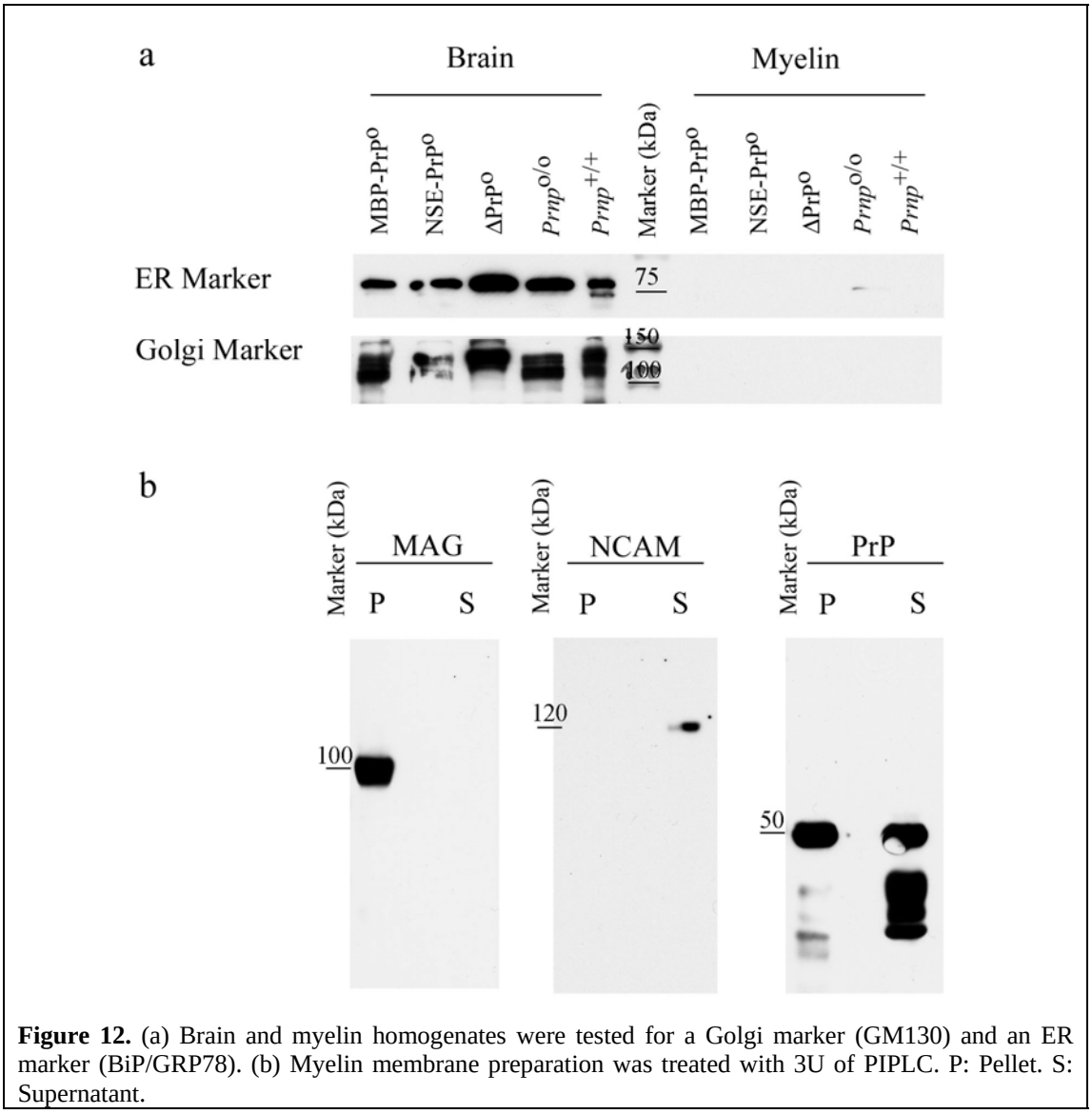


Figure 12. (a) Brain and myelin homogenates were tested for a Golgi marker (GM130) and an ER marker (BiP/GRP78). (b) Myelin membrane preparation was treated with 3U of PIPLC. P: Pellet. S: Supernatant.

Expression of myelin proteins is unchanged in ΔPrP^o mice

Leukoencephalopathy in ΔPrP^o mice exhibit features suggestive of compromised axon-glial adhesion, such as multiple intra-myelin splitting and axonal swelling. These alterations are similar to those described in mice deficient for different myelin proteins, such as MAG, PLP and CNP (Marcus et al 2002; Griffiths et al, 1998; Lappe-Siefke et al 2003). Therefore, we studied the expression levels of myelin proteins in ΔPrP^o mice.

We could not detect any striking differences in the protein profile of myelin preparations from whole brains from PrP-deficient, wild type and truncated PrP expressing mice, examined by silverstaining of an SDS-PAGE gel (Fig. 13, lanes 1-4).

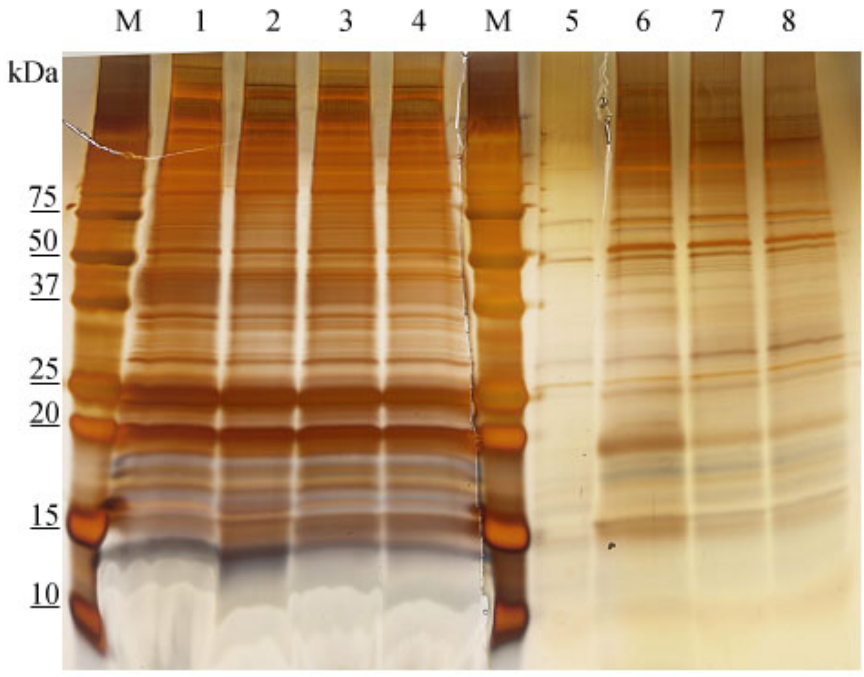


Figure 13. Silverstaining of myelin preparations (1-4) and of anti-PrP immunoprecipitations (5-6) of various genotypes. Myelin preparations of whole brain of *Prnp*$^{o/o}$ (1), wt (2), PrP$^{+/-}$ (3), and ΔPrP (4) mice. Immunoprecipitations of myelin preparations of Prnp$^{o/o}$ (5), wt (6), PrP$^{+/-}$ (7), and ΔPrP (8) mice. (M) Marker.

Additionally, we also investigated differences in protein levels in the different mouse genotypes by western blot for specific proteins such as myelin-associated glycoprotein (MAG), myelin basic protein (MBP) and proteolipid protein (PLP). No differences in protein expression between the different genotypes were revealed (data not shown).

PrP^C and ΔPrP interact with a restricted but heterogeneous group of myelin proteins

In order to identify PrP^C or ΔPrP binding partners in the myelin, anti-PrP antibody coated paramagnetic beads were used to precipitate PrP^C and ΔPrP in myelin preparations. Immunoprecipitations analyzed by a silverstained SDS-PAGE revealed that beads coated with anti-PrP antibodies incubated with myelin preparations from *Prnp*$^{o/o}$ mice non-specifically pulled down only a small number of proteins (Fig. 13, lane 5), emphasizing the specificity of the pull down. A restricted number of myelin proteins co-immunoprecipitated with PrP and ΔPrP (Fig. 13, lane 6-8) when compared to the number of proteins present in myelin inputs (Fig. 13, lane 1-4).

Western blot analysis of the immunoprecipitations identified four proteins that co-immunoprecipitated with PrP and ΔPrP. Two of them are proteins expressed in the non-compact, cytoplasm-containing myelin loops: 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP), and myelin associated glycoprotein (MAG). The other two proteins are localized in compact myelin: myelin basic protein (MBP) and myelin-associated/oligodendrocyte basic protein (MOBP) (Fig. 14). Whereas, proteolipid proteins (PLP), an integral "four-helix-span" membrane protein of the compact myelin, and myelin oligodendrocyte glycoprotein, a transmembrane protein of the non compact myelin, do not bind to PrP and ΔPrP, underlining the specificity of the interactions between the myelin proteins and PrP^C and ΔPrP.

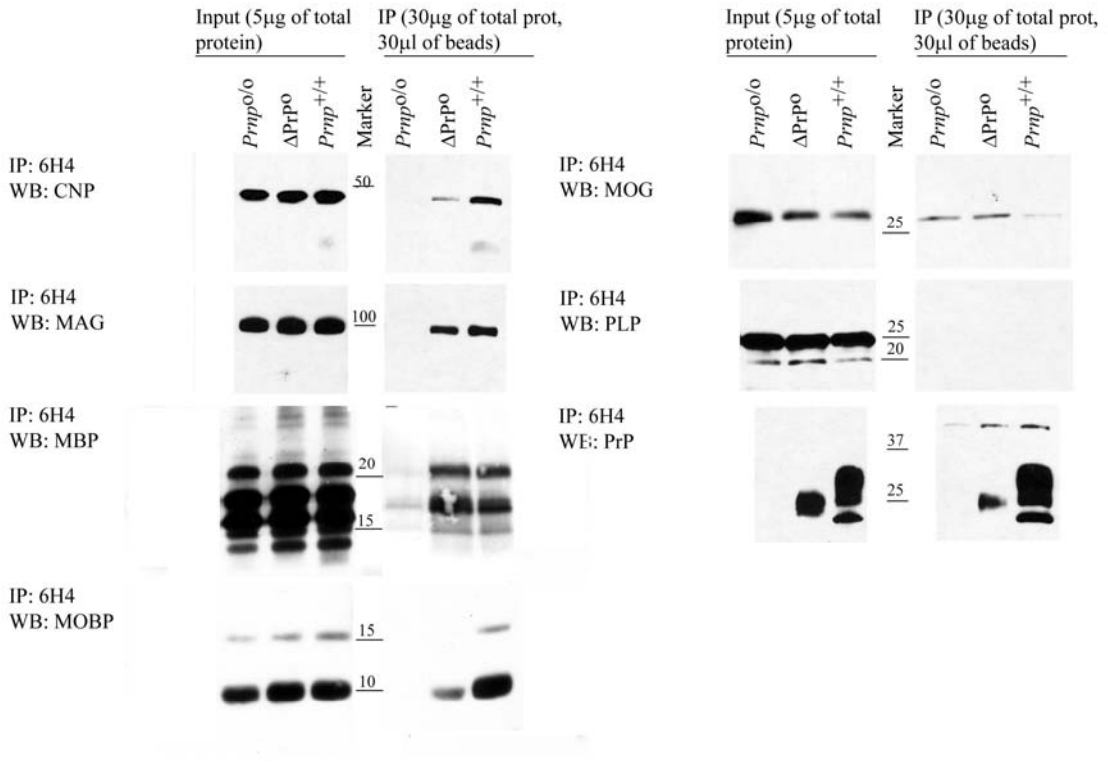


Figure 14. Pull downs with anti-PrP antibody coated beads co-immunoprecipitated CNP, MAG, MOBP and MBP with PrP^C and ΔPrP. No interactions are seen between PrP^C and ΔPrP, respectively, and MOG and PLP.

Interactions of PrP^C and ΔPrP with proteins expressed in myelin were confirmed by immunocytochemistry (Fig. 15) as well as co-immunoprecipitating them from spinal cord homogenate (data not shown). Confocal images show that CNP, MAG and MBP co-localize at least partially with PrP in oligodendrocytes of MBP-PrP mice. We used MBP-PrP mice for oligodendrocyte isolation, since oligodendrocytes from wild-type mice expressed PrP at undetectable levels for immunocytochemistry (Prinz et al., 2004). Oligodendrocytes isolated from NSE-PrP^o mice are deficient for PrP, as already shown by Western blot (Fig. 11B).

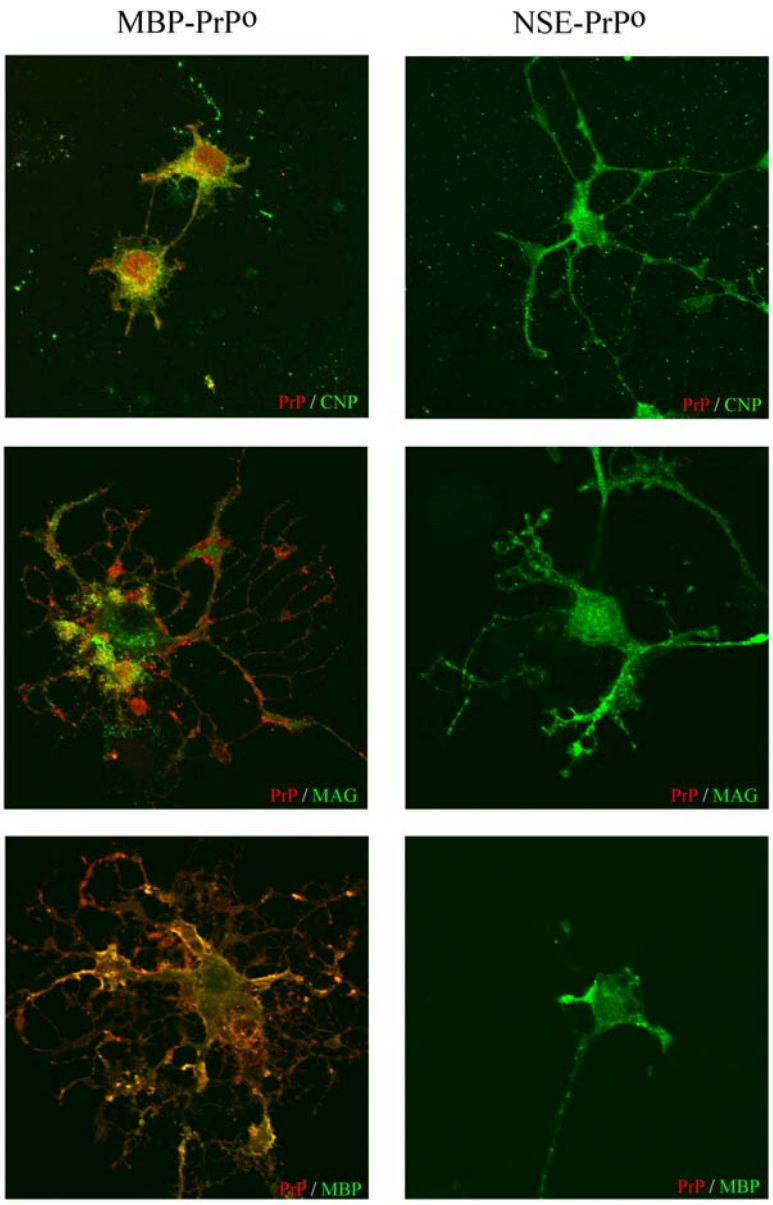


Figure 15. Mature murine oligodendrocytes cultured for 3 days and double immunostained for CNP, MAG or MBP (green) and PrP (red). MBP-PrP^{o} mice overexpress PrP in oligodendrocytes (Prinz et al., 2004), whereas NSE-PrP^{o} mice do not express PrP in oligodendrocytes.

ΔPrP is localized in lipid rafts

PrP^{C} is localized to lipid rafts (Meier et al., 2003; Naslavsky et al., 1997), where it may exert a physiological function by interacting with signaling molecules. If toxicity of ΔPrP results from interference with some PrP^{C}–mediated signaling pathway, and PrP^{C} counteracts toxicity, these two proteins may compete for the same partner in rafts. Moreover, ΔPrP may disrupt normal axon-glial adhesion and interaction, whose

integrity depend on GPI-anchored and transmembrane proteins (Bartsch, 2003; Falk et al., 2002; Marcus et al., 2002; Vinson et al., 2003).

We checked the topology of ΔPrP by performing flotation assays on OptiPrep gradient with Triton-X-100 (TX-100) treated brain homogenates from ΔPrP^{o} mice and with TX-100 treated myelin preparations from ΔPrP^{o} mice. The mutant PrP was detected in low-density, detergent insoluble fractions, and displayed buoyancy similar to that of PrP^{C} and flotilin (Fig. 16 and 17). These characteristics strongly suggest that ΔPrP is indeed localized in lipid rafts from total brain as well as from myelin and localized to the same rafts as PrP. Therefore, cytotoxicity is not due to inappropriate membrane targeting of ΔPrP.

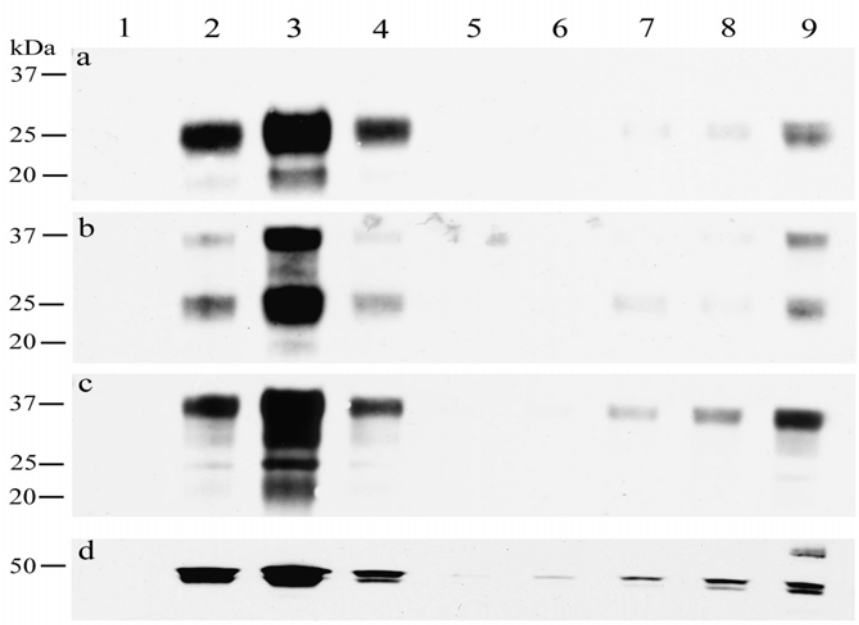


Figure 16. OptiPrep fractions of TX-100 treated brain homogenates were analyzed for PrP^{C} and ΔPrP expression on immunoblots. (a) ΔPrP^{o}, (b) ΔPrP^{+}, (c) wild-type tissues were analyzed. Mutant PrP^{C} localizes to the same rafts as PrP^{C}. (d) Flotilin, a 48kDa marker for rafts. Fractions 1-9, 1 the upper most fraction, 9 the bottom fraction.

The myelin proteins MAG and CNP co-localize with PrP^{C} and ΔPrP in CHAPS extracted lipid rafts

High cholesterol and glycosphingolipids in myelin suggests that rafts could be a major component of myelin and participate in the observed bi-directional signaling that occurs between oligodendrocytes/myelin and neurons (Sanchez et al., 1996; Vinson et al., 2003). Differences observed for the detergent solubility of specific myelin

proteins as a function of detergent-condition suggest a complex organization within rafts and that these detergent-specific DIGs may represent distinct biochemical, and possibly physiological, entities (Taylor et al., 2002a). Since PrP^C and ΔPrP are found in the same low-density fractions and interact with the same myelin proteins, differences in myelin protein partition in rafts might be responsible for ΔPrP toxicity. Therefore, we investigated if two, MAG and CNP, of the four found interaction partners for PrP^C and ΔPrP localize into the same rafts. We have chosen MAG and CNP, since similar phenotypes to the one observed in ΔPrP^o mice were observed in mice deficient for MAG and CNP.

We performed flotation assays on OptiPrep gradient with TX-100 and CHAPS extracted myelin preparation from ΔPrP^o mice and compared them to the ones from PrP^C and *Prnp*$^{o/o}$ mice. TX-100 and CHAPS were chosen, since it has been shown that MAG is solubilized by TX-100 (Vinson et al., 2003), but floats in lower density fractions when extracted with CHAPS (Taylor et al., 2002a). CNP has been shown to migrate within TX-100 as well as in CHAPS-insoluble fractions (Taylor et al., 2002a). PrP^C and ΔPrP from myelin preparations treated with either TX-100 or CHAPS floated to the same low-density fractions of the OptiPrep gradient (Fig. 17). CNP displayed also a significant insolubility in both detergents and floated to the same low-density OptiPrep fractions as PrP^C and ΔPrP. As was previously reported, MAG was soluble in TX-100 (Kim and Pfeiffer, 1999; Taylor et al., 2002a; Vinson et al., 2003). However, a significant amount of MAG is insoluble in CHAPS and floated to the same low-density fractions as PrP^C and ΔPrP. Thus, we can exclude any mistargeting of CNP and MAG in rafts due to ΔPrP. Both myelin proteins behave as described before.

The specific co-localization in CHAPS extracted rafts might explain the partial co-localized immunoreactivity for MAG and PrP observed in cultured oligodendrocytes (Fig. 15).

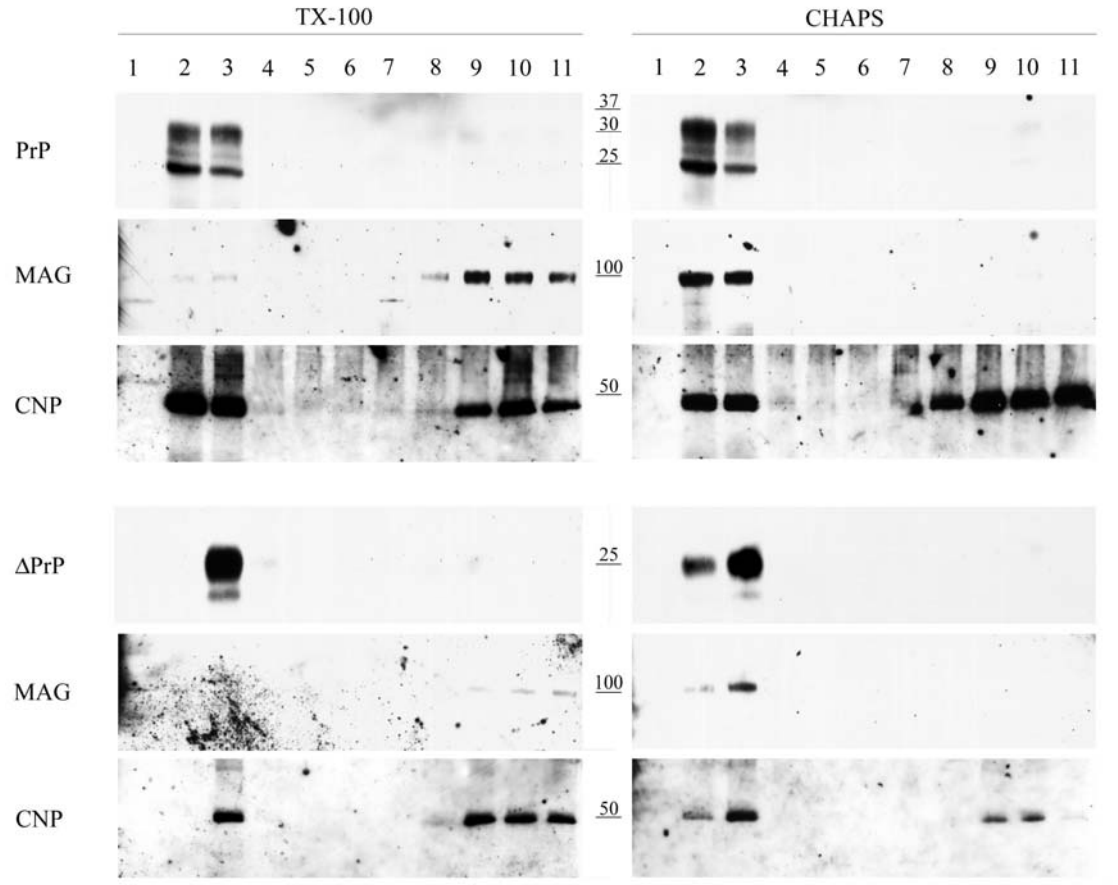


Figure 17. OptiPrep fractions of TX-100 and CHAPS-treated wild-type and ΔPrP^o myelin preparations. Western blot analysis of PrP, ΔPrP, MAG and CNP in TX-100 and CHAPS extracted myelin preparations on OptiPrep gradient, 1 representing the upper most fraction, and 11 the bottom fraction. Unstripped membranes were reused for immunoblotting with MAG and CNP, resulting in increased background.

Axonal p75^{NTR} does not rescue cerebellar granule cell degeneration nor white matter pathology in ΔPrP^o mice

Oligodendrocyte-specific expression of PrP^C rescues axonal degeneration in ΔPrP^o mice, suggesting an in *trans* interaction of PrP^C on oligodendrocytes with an axonal receptor. This interaction might elicit a survival signal in the axon. So far, we have probably investigated in *cis* interactions between PrP^C and ΔPrP, respectively, and myelin proteins.

A possible candidate for a *trans* interaction is the p75 neurotrophin receptor (p75^{NTR}). The PrP peptide (106-126), has been shown to interact with p75^{NTR} on CGCs (Della-Bianca et al., 2001). In addition, PrP^C and p75^{NTR} are both present in caveolar rafts of the plasma membrane (Bilderback et al., 1999; Peters et al., 2003), underlining a possible interaction. Besides its role in neurotrophic signaling, p75^{NTR} is implicated in

a diverse array of cellular responses, including apoptosis, neuronal survival, neurite outgrowth and myelination (Cosgaya et al., 2002; DeFreitas et al., 2001; Khursigara et al., 2001; Mi et al., 2004). So far, it is unknown whether ΔPrP toxicity represents a gain-of-function, e.g. induction of cell death, or a loss-of-function, e.g. absence of a survival signal.

To investigate if the interaction between $p75^{NTR}$ and ΔPrP could be responsible for cerebellar neurodegeneration and/or axonal pathology in ΔPrP^{o} mice, we crossed ΔPrP^{o} mice with $p75^{NTR}$ deficient mice (Lee et al., 1992) (referred to as $p75^{o/o}$; ΔPrP^{o} mice). The phenotype of mice lacking $p75^{NTR}$ ($p75^{o/o}$ mice) consists of a smaller size than their wild-type littermates and of an abnormal waddling gait.

The ablation of $p75^{NTR}$ in ΔPrP^{o} mice, however, neither delayed nor rescued the defects, characterized by ataxia, hind leg paralysis and wasting (Fig. 18). The ΔPrP^{o} mice devoid of $p75^{NTR}$ died at 86 ± 8 days, whereas ΔPrP^{o} mice expressing $p75^{NTR}$ died around 100 ± 11 days.

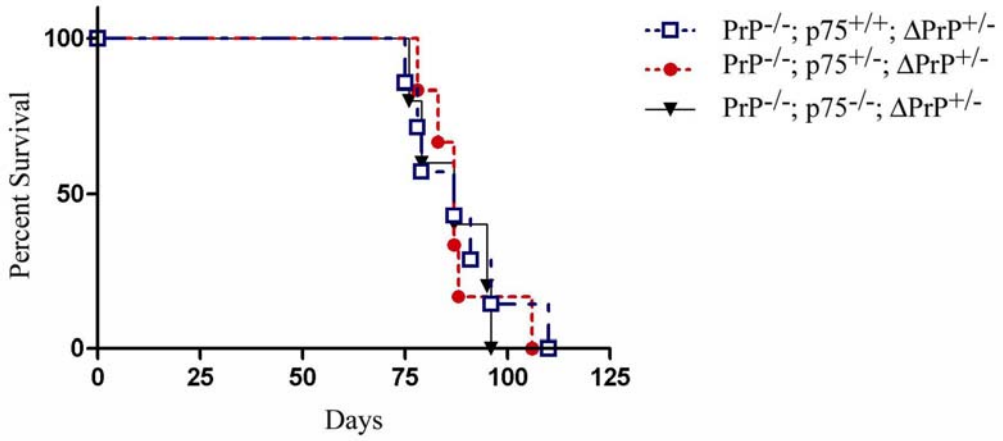


Figure 18. Comparison of survival of $p75^{o/o}$; ΔPrP^{o} mice to ΔPrP^{o} mice.

Histological analysis revealed massive cerebellar degeneration in ΔPrP^{o} as well as in $p75^{o/o}$; ΔPrP^{o} mice (Fig. 19 a, b, e, f,). The faint stainings for Luxol-Nissl and neurofilament 200 (NF-200) indicate that important myelin and axonal degeneration, respectively, has occurred (Fig. 19 i, j, m, n), whereas ΔPrP^{+} and $p75^{o/o}$ mice show normal cerebellar architecture as well as strong staining for myelin (Luxol-Nissl) and axons (NF-200).

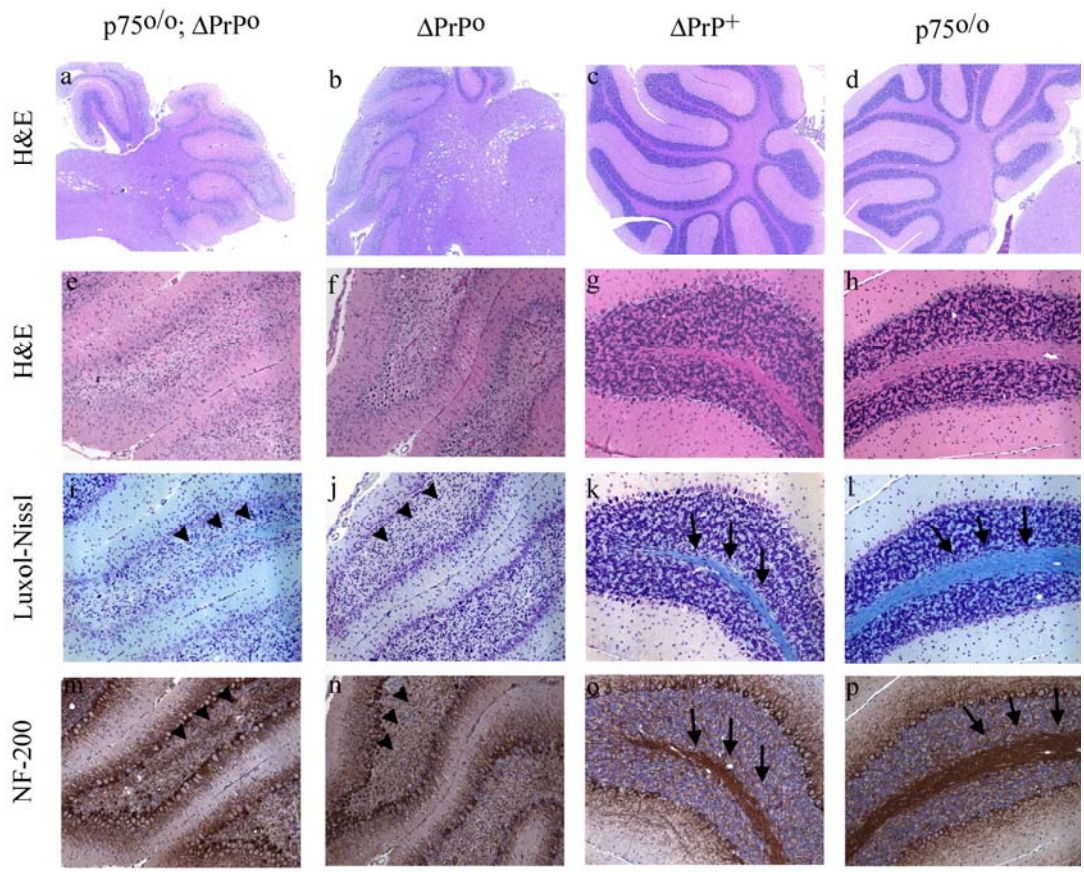


Figure 19. Histology of cerebella from p75$^{o/o}$; ΔPrP^{o}, ΔPrP^{o}, ΔPrP^{+}, and p75$^{o/o}$ mice. (a-d) H&E staining. Magnification 25x. (e-h) HE staining. Magnification 100x. (i-l) Luxol-Nissl staining. Arrowheads indicate the weakened staining for myelin in sick mice and arrows the normal staining for myelin in healthy mice. Magnification 100x. (m-p) NF-200 staining. Arrows indicate the weakened staining for axons in sick mice and arrows the strong staining in healthy mice. Magnification 100x.

DISCUSSION

ΔPrP induces white matter pathology

Overexpression of amino terminally truncated PrP^C variants collectively termed ΔPrP triggers not only neuronal degeneration (Shmerling et al., 1998), but also leukoencephalopathy. This pathology was unexpected, since PrP-related diseases are thought to affect primarily neurons (Chiesa et al., 1998; Hegde et al., 1998; Hegde et al., 1999; Rossi et al., 2001; Shmerling et al., 1998). The white matter pathology displayed features suggestive for compromised axon-glial adhesion similar to those described in mice lacking myelin-associated glycoprotein and myelin galactolipids (Marcus et al., 2002), as well as in mice deficient for proteolipid protein (Griffiths et al., 1998). In addition, we found axonal swellings similar to those of *Cnp1*$^{o/o}$ mice (Lappe-Siefke et al., 2003).

Myelinopathy, rather than neuronal damage, may be a major determinant of lethality in Shmerling's syndrome, since oligodendrocyte-restricted expression of PrP^C selectively suppresses leukoencephalopathy, and restores long-term survival. Instead, neuron-restricted expression of PrP^C, even at supraphysiological levels, does not prevent white matter degeneration

Rescue of white matter pathology by oligodendroglial PrP^C expression

Oligodendrocyte-restricted expression of PrP^C in ΔPrP^o selectively suppresses leukoencephalopathy, whereas, neuron-specific expression of PrP^C rescues partially cerebellar neurodegeneration, but not white matter disease. The presence of PrP^C either in neurons or in oligodendrocytes restores in both cases long-term survival. Complete rescue, however, is only brought about by endogenous PrP^C expression, which occurs on both neurons (Kretzschmar et al., 1986) and glia (Moser et al., 1995). The selective rescue of leukoencephalopathy in ΔPrP^o mice by full-length PrP on oligodendrocytes indicates that PrP^C restores in *trans* the axonal pathology elicited by mutant PrP. This suggests that PrP^C transduces a cellular signal from oligodendrocytes to axons, analogous to other GPI anchored proteins (such as F3 and NCAM120) that play a role in axon-myelin interactions (Falk et al., 2002). As PrP^C

binds in vitro to the transmembrane and GPI-linked forms of N-CAM (Schmitt-Ulms et al., 2001), it might contribute to the very same pathways. Alternatively, PrP^C may instruct oligodendrocytes to release factors necessary for axonal survival (Wilkins et al., 2003).

Surprisingly, neuron-specific PrP^C expression did not completely rescue CGC degeneration. Perhaps ongoing degeneration of white matter fibers innervating the granule cell layer participated indirectly in granule cell degeneration, e.g. by deafferentiation and neuronal "dying back". The delayed onset of myelinopathy in ΔPrP^o; NSE-PrP mice might be related to the presence of PrP^C in NSE-PrP myelin, which, in turn, might be due to GPI cell painting (Anderson et al., 1996).

PrP^C present in myelin interacts with myelin proteins

Similar phenotypes in mice deficient for myelin proteins such as MAG, PLP and CNP (Griffiths et al., 1998; Lappe-Siefke et al., 2003; Marcus et al., 2002) suggested a possible implication of these proteins in the myelinopathy observed in ΔPrP^o mice. Yet, we have not found any evident changes in expression levels for any of these proteins.

We could show that PrP^C as well as ΔPrP are present in myelin. The full-length as well as the mutant prion protein seem to interact specifically with different myelin proteins: MAG and CNP situated in the non-compact myelin; and MBP and MOBP, localized to the compact myelin. Co-localization studies on mature primary oligodendrocytes revealed at least a partial overlapping signal for PrP and MAG, CNP and MBP, respectively, pointing out the specificity of the observed interactions.

PrP^C is localized to lipid rafts (Meier et al., 2003; Naslavsky et al., 1997), where it may exert a physiological function by interacting with signaling molecules. Lipid rafts have been implicated in membrane signaling (Anderson, 1993; Lisanti et al., 1994; Parton and Simons, 1995). If toxicity of ΔPrP results from interference with some PrP^C–mediated signaling pathway, and PrP^C counteracts toxicity, these two proteins may compete for the same partner on rafts. We first checked if the mutant PrP is also localized to lipid rafts. TX-100 treated brain homogenates and myelin preparations of ΔPrP^o mice were subjected to gradient centrifugation. ΔPrP floated to the same low-density, detergent insoluble fractions as the full-length prion protein, underlining our

hypothesis and excluding that neurotoxicity of ΔPrP is due to membranal mistargeting. We then investigated the association of the myelin proteins MAG and CNP with lipid rafts. We compared TX-100 and CHAPS treated myelin preparations from wild-type and ΔPrP^{o} mice. MAG has been reported to be solubilized in TX-100. Whereas when treated with CHAPS (a zwitterionic detergent structurally resembling cholesterol), this protein floats to low-density, detergent insoluble fractions. Our results confirmed these recent findings. PrP, ΔPrP and CNP when extracted by TX-100 or CHAPS migrated to low-density fractions. Thus, PrP^{C} and ΔPrP, respectively, and MAG and CNP shared the same rafts only, when rafts were extracted with CHAPS. As discussed by Taylor and colleagues (Taylor et al., 2002a), differentially extracted rafts may represent biochemically, eventually physiologically relevant, entities.

The interaction between PrP^{C} or ΔPrP and the non-compact myelin proteins MAG and CNP

Myelin-associated glycoprotein (MAG) is a minor, but important constituent of myelin, comprising 1% of all myelin proteins in the CNS (Trapp, 1990). It is an integral membrane glycoprotein (Poltorak et al., 1987; Yin et al., 1998) and member of the immunoglobulin gene superfamily (Salzer et al., 1987). When compact myelin has formed, expression of MAG becomes restricted to the periaxonal region of the myelinating cell (Sternberger et al., 1979), which is consistent with its known function in glial-neuronal interactions (Domeniconi et al., 2002; Franzen et al., 2001; Vyas et al., 2002). *Mag* knockout mice produce relatively normal myelin, but develop late-onset degeneration of axons and myelin (Fruttiger et al., 1995; Yin et al., 1998).

The extracellular domain of MAG predisposes the molecule for interactions with ligands and receptors (Schachner and Bartsch, 2000). Moreover, it has been suggested that the extent of MAG-mediated interactions in *trans* (i.e. between two apposing cell membranes) is regulated in *cis* (i.e. within the same cell membrane) by sialic acid containing constituents (Tropak and Roder, 1997). We have shown that PrP^{C} expressed on oligodendrocytes suppresses axonal degeneration, indicating that PrP^{C} rescues the pathology by an interaction in *trans* with an axonal molecule. Alternatively, our co-immunoprecipitation studies in myelin suggest *cis* interactions with myelin proteins.

Since the prion protein contains two N-linked glycosylation sites that carry sialic acids, a *cis* interaction between MAG and PrP becomes likely. This *cis* interaction might enhance the in *trans* interaction of MAG with an axonal receptor, giving rise to survival signal for the axon. MAG has been shown to interact with Nogo66 receptor on neurons (Domeniconi et al., 2002) that may initiate a bi-directional signaling system that results in inhibition of neurite outgrowth postnatally and maintenance of myelin integrity. The inhibition of neurite outgrowth is probably mediated via activation of Rho A kinase, whereas the maintenance of myelin integrity is probably regulated via the activation of Fyn kinase (Vinson et al., 2001; Vyas et al., 2002; Wong et al., 2002; Yamashita et al., 2002).

Crosslinking of MAG (Umemori et al., 2002) and PrP (Mouillet-Richard et al., 2000) with specific antibodies have been shown to result in activation of Fyn kinase, suggesting a common signaling pathway. Because PrP^{C} is GPI-linked to the neuronal surface, its lack of cytoplasmic domain suggests the participation of an interacting transmembrane receptor subunit that activates Fyn. Activation of Fyn might elicite a signaling pathway that instructs oligodendrocytes to maintain myelin integrity and release trophic factors to support axons. Given the association between MAG and PrP, it is tempting to speculate that such a signaling cascade might involve MAG.

The N-terminally truncated PrP still interacts with MAG, but the missing flexible N-terminal part might hinder a functional interaction leading to an abnormal signaling – either suppressed or enhanced – leading to the phenotype observed in ΔPrP^{o} mice (Fig. 4).

2', 3'-Cyclic nucleotide 3'phosphodiesterase (CNP) exists in two isoforms of 46 and 48 kDa. It accounts for approximately 4% of all CNS myelin protein and is distributed throughout the cell soma (Nishizawa et al., 1985) and in non-compacted regions of myelin (Braun et al., 1988; Trapp et al., 1988). Both proteins are acylated (Braun et al., 1991; De Angelis and Braun, 1994), which explains their efficient association with the cytoplasmic cellular membrane. It is, therefore, not evident how the interaction between full-length PrP or the mutant PrP, both GPI-anchored proteins situated at the outer surface of the membrane, and CNP, situated at the inner leaflet of the membrane, occurs. Perhaps, CNP binds indirectly via a third molecule, e.g. MAG, to PrP. In this complex, CNP might act as a downstream effector.

The function of CNP does not shed light on the importance and significance of the interaction between PrP^C and CNP. It has been shown to hydrolyze 2'3'-cyclic nucleotides into their 2'-derivatives. However, because 2'3'-cyclic nucleotides have not been found in the brain, the function of CNP remains obscure. CNP influences cell morphology (De Angelis and Braun, 1994) and is thought to be a regulator of events leading to myelination (Braun et al., 1991), so the finding that *Cnp1* knockout mice developed myelin that was biochemically, ultrastructurally, and morphologically normal was unexpected (Lappe-Siefke et al., 2003). However, at about 4 months of age, the mice developed motor deficits that progressed with age. Histological analysis revealed late-onset axonal pathology characterized by abnormal axonal swellings and degeneration of many axons, clearly not related to dys- or demyelination (Lappe-Siefke et al., 2003). In these mice, axonal degeneration is not caused by abnormal myelination but constitutes a primary phenotype of CNP deficient-mice.

The interaction between PrP^C or ΔPrP and the compact myelin proteins MBP and MOBP

Myelin basic protein is one of the major proteins of the CNS myelin and constitutes as much as 30% of myelin proteins. In fact, it is a family of proteins, as there are many isoforms of different molecular masses. The molecular masses of the major forms are 21.5, 18.5, 17 and 14 kDa in the mouse. In the adult, the predominant isoforms are 18.5 and 14 kDa, constituting 95% of the MBPs. MBP is bound to the cytosolic side of the oligodendrocyte membrane, primarily through electrostatic interaction with acidic lipids. Direct evidence that MBPs play a major role in myelin compaction in the CNS was provided from studies of the shiverer mutant mouse, which presents a large deletion of MBP gene (Roach et al., 1985) and in which the major dense line is absent from myelin (Privat et al., 1979).

The interactions seen between MBP and PrP^C or ΔPrP seem to be specific. However, these interactions lack evidence about how the interaction takes place, since PrP and MBP are placed opposite each other on the membrane. As MBP has been shown to bind to CNP (Harauz et al., 2004), it might form a complex with PrP, MAG, and CNP, in which MBP takes over the role in signal transduction. Indeed, it has been proposed that MBP is involved in signal transduction (Dyer, 2002; Dyer et al., 1994).

Since ΔPrP^{o} mice do not show any abnormalities in myelin periodicity and spacing, PrP^{C} seems not to be involved in compaction of myelin.

Myelin-associated oligodendrocyte basic protein (MOBP) is, like MBP, a highly basic and positively charged protein (Yamamoto et al., 1994). Six splice variants have been identified which are predicted to encode five different protein isoforms of MOBP (Holz et al., 1996; Yamamoto et al., 1994). Though all isoforms so far identified have amine acid residues 1-68 in common, they differ in the length and polarity of their respective C-terminal regions (Holz et al., 1996; Montague et al., 1997) that contains a cluster of positively charged residues. These positively charged amino acids are considered to interact with phospholipids in the inner surface of the plasma membrane, and are concentrated where the cytoplasmic faces of the myelin sheath are in apposition, leading to the formation of myelin dense lines (MDLs) together with MBP. Since MOBPs are located in the MDL of myelin, they could play a similar role to MBP in myelin compaction (Holz and Schwab, 1997). However, MOBP-deficient mice exhibit no obvious phenotype (Yamamoto et al., 1999; Yool et al., 2002), suggesting that it is not essential for forming compact myelin. Thus, its physiologic function remains unclear. Although the association between PrP^{C} and ΔPrP with MOBP seemed to be specific, its biological relevance remains enigmatic.

During purification of the PK-resistant core of PrP^{Sc} (PrP^{27-30}), several molecules have been identified that co-purified with PrP^{27-30} and prion infectivity (Stahl et al., 1993). It is unknown whether these molecules simply represent contaminants with physical properties similar to PrP^{27-30}, or whether they participate as components of the prion. These molecules included peptides with sequences DGPRLSK and REIVDRK. The source of these peptides was not known at that time, but performing a database search, we retrieved one protein containing both peptide sequences, MOBP. We found that MOBP is resistant to PK (data not shown), explaining partially its ability to co-purify with PrP^{27-30}. If MOBP plays a role in prion disease propagation or transport is currently under investigation.

Trans-interaction of PrP with an axonal protein

The interactions between PrP^{C} or ΔPrP and the myelin proteins most probably occur in *cis*. However, the rescue of myelinopathy in ΔPrP^{o} mice by oligodendrocytes-

specific expression of PrP^C suggests an interaction in *trans* with a receptor or a ligand on the axon.

PrP has been shown to act as a trans-interacting partner for neurons and to promote neuronal survival and neurite outgrowth (Chen et al., 2003; Kuwahara et al., 1999). Recently, the p75 low affinity NGF receptor ($p75^{NTR}$) has been reported to bind a prion protein fragment, PrP (106-126). In addition, PrP^C and $p75^{NTR}$ are both present in caveolar rafts of the plasma membrane (Bilderback et al., 1999; Peters et al., 2003), underlining a possible interaction. $p75^{NTR}$ is implicated, besides its role in neurotrophic signaling, in a diverse array of cellular responses, including apoptosis, neuronal survival, neurite outgrowth and myelination (Cosgaya et al., 2002; DeFreitas et al., 2001; Frade et al., 1996; Khursigara et al., 2001; Mi et al., 2004). Although it may seem curious that $p75^{NTR}$ signaling causes both cell death and cell survival, this is the case for other members of the TNF receptor superfamily (Nagata, 1997). $p75^{NTR}$ has been shown to interact with Nogo-66 receptor and mediate growth cone and neurite outgrowth inhibition, the signaling of which are elicited by the binding of MAG, Nogo-A and OMgp to the Nogo-66 receptor (Wang et al., 2002).

Based on these findings, we have speculated that an interaction between ΔPrP and $p75^{NTR}$ could elicit a signal leading to cell death and axonal pathology. Therefore, we bred $p75^{NTR}$ deficient mice with ΔPrP^o mice in order to see if axonal pathology and cerebellar granule cell degeneration are rescued by the ablation of $p75^{NTR}$.

However, disease onset and lifespan of $p75^{o/o}$; ΔPrP^o mice did not differ from ΔPrP^o mice. Histological analysis revealed a thinned cerebellar granule cell layer due to neuronal degeneration and coarse vacuolation in white matter. Moreover, Luxol-Nissl staining was significantly weakened in the $p75^{o/o}$; ΔPrP^o mice cerebellum and spinal cord of $p75^{o/o}$; ΔPrP^o mice compared to wild-type mice, indicating myelin degeneration. Axon degeneration also occurred, as evidence by a faint NF-200 staining. p75 receptor deficient mice, that we have used in our studies, still expressed a shorter splice variant that is unable to bind neurotrophins (Lee et al., 1992). Incomplete ablation leads to a phenotype in the peripheral nervous system with hypomyelination and a decrease in the number of axons (Cosgaya et al., 2002). Phenotypically, the mice are smaller than their littermates and show a waddling gait. In contrast, in ΔPrP^o mice, we have not observed any pathology in the peripheral nervous system. In the $p75^{o/o}$; ΔPrP^o mice, the sciatic nerve is macroscopically thinner

probably due to the described decrease in number of axons and hypomyelination (Lee et al., 1992; von Schack et al., 2001). However, this additional pathology has no influence on the course of the disease. Complete ablation of all $p75^{NTR}$ isoforms produced a larger decrease in the number of neurons and Schwann cells present in the sciatic nerve compared with the traditional $p75^{NTRo/o}$ mice (von Schack et al., 2001). In addition, the absence of both isoforms leads to partial perinatal lethality and defects in the vascular systems. This suggests an additional neurotrophin-independent role for this receptor and/or redundant function of the isoforms. The incomplete ablation might be insufficient to rescue ΔPrP-induced pathology. However, more likely, the observed *in vitro* interaction between PrP and $p75^{NTR}$ is not relevant *in vivo.*

PrP^C seems to play a role in myelin physiology

The white matter pathology in ΔPrP^o mice displayed features suggestive of compromised axon-glial adhesion, such as intra-myelin splitting and axonal swellings. These changes are similar to those described in mice lacking MAG and myelin galactoplipids (Marcus et al., 2002), and PLP (Griffiths et al., 1998). In addition, we found axonal swellings similar to those of *Cnp1*$^{o/o}$ mice (Lappe-Siefke et al., 2003).
These data from myelin protein gene null mice highlight the dual role of the oligodendrocytes: first, in formation of the myelin sheath and, second, in supporting the underlying axon. The axon-myelin complex may be a primary target of ΔPrP. The finding, that full-length prion protein represses the defects induced by the mutant prion protein, suggests that PrP^C plays a role in myelin physiology, in oligodendroglial-mediated axonal support and axon-myelin interaction. The interaction of PrP^C with a variety of myelin proteins underscores this possible role. A delay in initiation of myelination was excluded as a cause for the observed white matter disease in ΔPrP^o mice. Abnormal myelin structure was also ruled out as a reason for myelin and axonal degeneration, further supporting the importance of PrP^C in axon-glial interaction. The late onset of axonal degeneration seen in ΔPrP^o mice indicate that axonal requirements change over time and that the factors required for the development and maintenance of the myelinated axon are different or deficits in myelin-axonal signaling accumulate until a threshold is reached whereupon axonal degeneration begins. A possible role for PrP^C in myelin maintenance is further

supported by demyelination of the peripheral nervous system in the PrP null mouse, that provokes no phenotype but is corrected by PrP expression (Nishida et al., 1999).
However, the biological and physiological relevance of the interactions between PrP^C, or ΔPrP, and myelin proteins remain to be proven. ΔPrP might sequester myelin proteins, that seem to be necessary for axonal support, or ΔPrP might interfere with the physiological function of one of the identified myelin proteins, consisting of signal transduction or axon-glial interaction. How myelin proteins act on the health of an axon remains unclear. Recently, Menon and colleagues (Menon et al., 2003) have reported that high-affinity protein-protein interactions occur between myelin and the axolemma, which may be potential sites for bi-directional signaling. Or, myelin proteins exert a trophic action on the axon, directly or indirectly mediated by downstream factors.

Ongoing studies to reveal the biological relevance of the interactions between PrP or ΔPrP and myelin proteins

In vivo and *in vitro* studies support a trophic role for oligodendrocytes on nearby neurons. Conversely, expression of growth factors by oligodendrocytes is influenced by neural signals.
In vivo maturation of axons, as indicated by neurofilament development (Sanchez et al., 1996) or the clustering of sodium channels (Kaplan et al., 1997), is correlated with oligodendrocyte ensheatment. Additionally, myelin proteins such as PLP and CNP have been implicated in axonal support (Griffiths et al., 1998; Lappe-Siefke et al., 2003).
Identified trophic factors that may be responsible for trophic support include: BDNF, NT-3 (Dai et al., 1997) and IGF-1 (Wilkins et al., 2001). These factors have been shown to be produced by oligodendrocytes. In addition to the expression of neurotrophins and IGF-1, recent literature suggests that oligodendrocytes produce neuregulins and GDNFs, as well as transforming growth factor-βs, and fibroblast growth factor-9 (Du and Dreyfus, 2002).
Interaction between PrP and myelin proteins might lead to activation of a signaling pathway that instructs oligodendrocytes to secret such trophic factors. In the case of ΔPrP, this signal might be missing and the absence of the trophic factor eventually leads to axonal pathology.

To test for oligodendrocytic dysfunction due to ΔPrP expression, we are currently establishing an *in vitro* model. Primary oligodendrocytes of ΔPrP^{o} mice are cultured in serum-free medium. This medium is then transferred onto neurons, e.g. primary cerebellar granule cells or dorsal root ganglia, to test for its ability to induce axonal degeneration (Wilkins et al., 2003). Another approach using co-cultures of DRGs and oligodendrocytes isolated from ΔPrP and wild-type mice might also be used to check for oligodendrocytic dysfunction and enhanced axonal degeneration.

To test the relevance of the association between PrPs and myelin proteins *in vivo*, we are breeding the different myelin protein deficient mice with $Prnp^{o/o}$ and ΔPrP^{o} mice. If PrP^{C} has a role in myelin maintenance or axonal support, we might see an enhanced phenotype in the double knockout mice. Often the ablation of one myelin protein does not lead to a characteristic phenotype, however, the deletion of two myelin proteins often induces an enhanced phenotype (Biffiger et al., 2000; Marcus et al., 2002; Uschkureit et al., 2000). If one of the myelin proteins is important in mediating an aberrant signaling due to the interaction with ΔPrP^{o}, we might even rescue the pathology in ΔPrP^{o} mice. Furthermore, we inoculated small groups of $Mag^{o/o}$ (Montag et al., 1994) and $Mobp^{o/o}$ (Yool et al., 2002) mice intracerebrally or intranervally with high and low dose of scrapie prion (Rocky Mountain Laboratory (RML) strain, passage 5), in order to see if one of the myelin proteins could be important for the transport or replication of the infectious prion protein. *Cnp* deficient mice (Lappe-Siefke et al., 2003) as well as *Mbp* deficient mice (Chernoff, 1981) are not suitable for inoculation, since they have a limited lifespan of 210-330 and 90-150 days, respectively. The mechanism, which is involved in prion spread to and within the CNS, is not yet clear. There are data that support the hypothesis of axonal prion transport (Brandner et al., 1996b; Fraser, 1982). Other findings, however, suggest nonaxonal transport mechanisms that result in periaxonal deposition of PrP^{Sc} (Glatzel and Aguzzi, 2000; Hainfellner and Budka, 1999). One might hypothesize a “domino” mechanism, by which incoming PrP^{Sc} converts resident PrP^{C} on the axolemmal or myelin surface, thereby spatially propagating the infection (Aguzzi and Weissmann, 1997). Mice that express PrP^{C} selectively in oligodendrocytes have been shown to be resistant to prion infection, ruling out that oligodendrocytes replicate and propagate PrP^{Sc}. Therefore, PrP^{C} expressed on myelin surface can be excluded to be important for prion spread. However, one could speculate that other proteins on the myelin

surface help to convert and propagate prions. Since myelin proteins have been implicated as regulators of axonal transport (de Waegh and Brady, 1990; Kirkpatrick et al., 2001), myelin proteins might also be implicated in prion spread.

Conclusion

Our data are clearly consistent with a function of PrP^C in myelin physiology and axon-glial interaction. The interaction with myelin proteins in this context is plausible. However, further experiments are necessary to sustain their biological relevance. In addition, we cannot rule out the importance of a high-affinity interaction of PrP^C with an axonal protein complex that gives rise to a survival signal. ΔPrP seems to abrogate this function of myelin maintenance and axon-glial interaction and therefore is a suitable model to test for the importance of the interaction *in vivo* and *in vitro*.

An *in vitro* model to dissect mechanisms leading to cell death induced by amino-terminally truncated prion protein and Doppel

RESULTS

Lentiviral constructs

The cDNA of *Prnp, ΔPrnp and Prnd* were fused to an internal ribosomal entry site (IRES) controlling the expression of green fluorescent protein (GFP) (e.g. Dpl-IRES-GFP). From these constructs a bicistronic mRNA encoding the cDNA of interest and GFP is transcribed that provides GFP levels as a surrogate marker for cDNA expression and allows for quantification of transduced cells by fluorescence-activated flow cytometry (FACS). These constructs were then cloned into a 3rd generation lentiviral transfer vector, pCCLsin.PPT.CMV.eGFP.Wpre (Follenzi and Naldini, 2002) (Fig. 20). The envelope construct, pMD.G, expressing the surface glycoprotein VSV.G, and the packaging construct, pCMVΔR8.91, encoding the proteins and enzymes of the vector core, were from the 2nd generation of lentiviral vectors (Fig. 20).

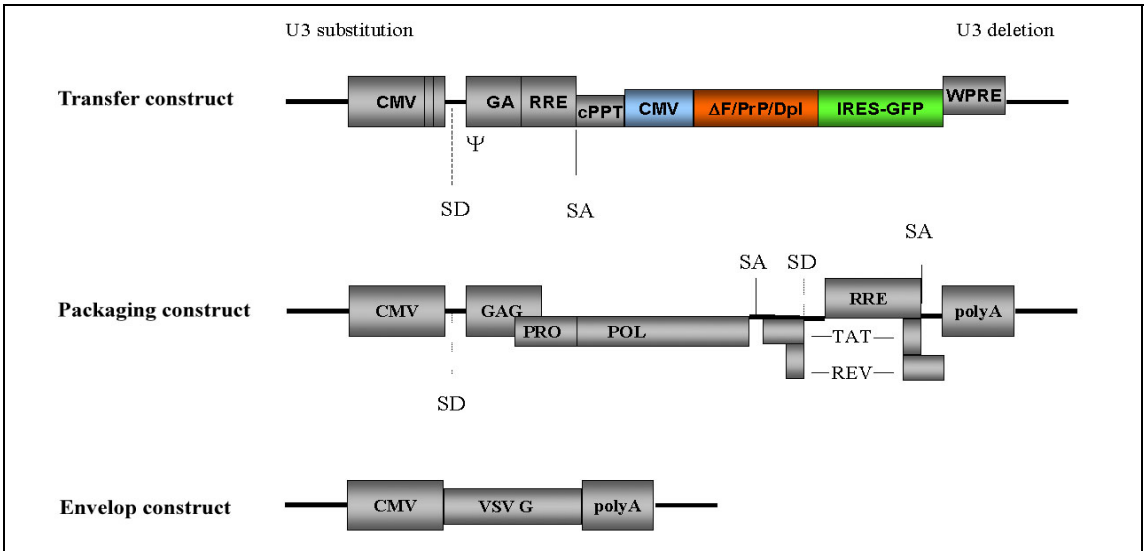


Figure 20. The three-plasmid vector system used to generate the different lentiviral vectors. SA and SD Splice acceptor and donor sites, ψ the packaging sequence, GA 5' portion of gag gene with truncated reading frame including extended packaging signal, RRE Rev Response Element, PolyA polyadenylation site, cPPT central polypurine tract, Wpre posttranscriptional regulatory element from the genome of the woodchuck hepatitis virus, VSV.G glycoprotein of vesicular stomatitis virus, which gives the vector a wide host range (adapted from Follenzi and Naldini (Follenzi and Naldini, 2002)).

Assaying transducing activity

To verify transducing activity of vector stocks, we performed end-point titration on T293 cells and analyzed them subsequently by FACS. Figure 21 illustrates FACS analysis of T293 cells that were transduced with the constructs of CMV.eGFP and of CMV.Dpl.IRES.eGFP. The titers of the constructs containing the different cDNAs were always lower than the titers of the eGFP construct. The lowest dilution of the eGPF lentivirus (independent productions) yielded at least 75% of eGFP positive cells, whereas the virus of PrP, ΔPrP and Dpl virus produced approximately 25% of eGFP positive cells. Nevertheless, the titers for PrP, ΔPrP and Dpl virus were still in the expected range of 10^8-10^9 transducing units (TU)/ml.

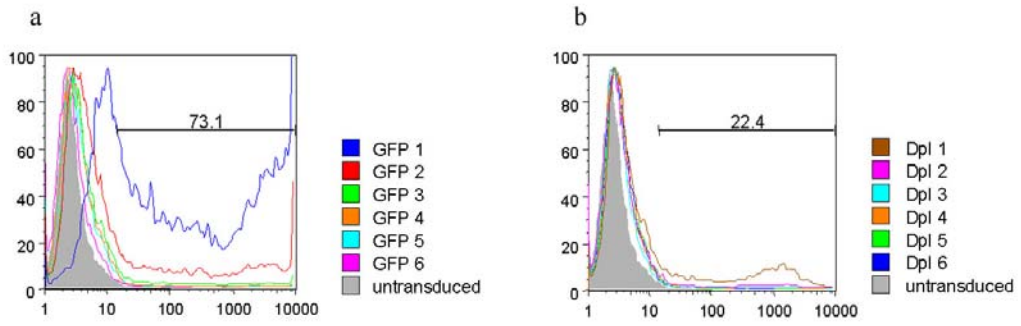


Figure 21. Titration of eGFP and Dpl lentivirus on T293 cells. (a) T293 cells transduced with eGFP lentiviral construct. GFP 1 - GFP 6: dilution from 10^{-1} - 10^{-6}. (b) T293 cells transduced with Dpl lentiviral construct. Dpl 1 - Dpl 6: dilution from 10^{-1} - 10^{-6}.

In addition, we compared IRES.eGFP expression to PrP expression by labeling the cells with an anti-PrP antibody (6H4) and a Cy3–conjugated secondary antibody. We gated for endogenous PrP expressed by T293 cells in non-transduced cells and compared them to T293 cells transduced with different concentrations of lentiviral vector expressing the full-length prion protein (Fig. 22). Based on the FACS data, we can conclude that eGFP expression and PrP expression coincide. Therefore, eGFP based end-point titration was used for titer calculation.

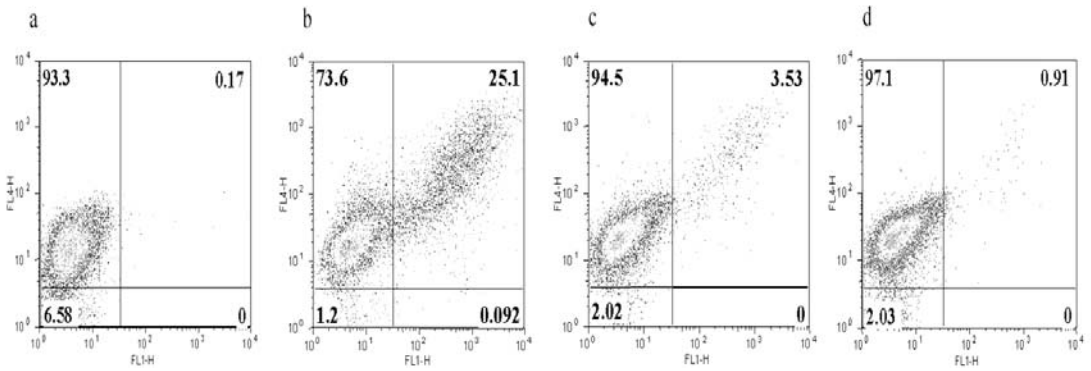


Figure 22. Comparison between PrP and eGFP expression in T293 cells. (a) Endogenous PrP expression in untransduced T293 cells. Expression of PrP and eGFP in T293 cells transduced with three different dilutions of PrP lentivirus: (b) 10^{-1} (c) 10^{-2} (d) 10^{-3}. FL4-H: signal for cy3 antibody. FL1-H: signal for eGFP.

Expression of the different proteins from lentiviral transduced murine CGCs

CGCs were transduced with various amounts of the different lentiviral vector stocks. The cells showed a visually detectable GFP expression 2 days after transduction. After three days, the cells were lysed and the expression levels of each vector were evaluated on Western blots (Fig. 23a). All three proteins were expressed at reasonable levels and showed an expression - lentivirus dose correlation. Figure 23b illustrates the expression of ΔPrP over time. The protein can be detected as early as 33h after transduction of the primary neurons.

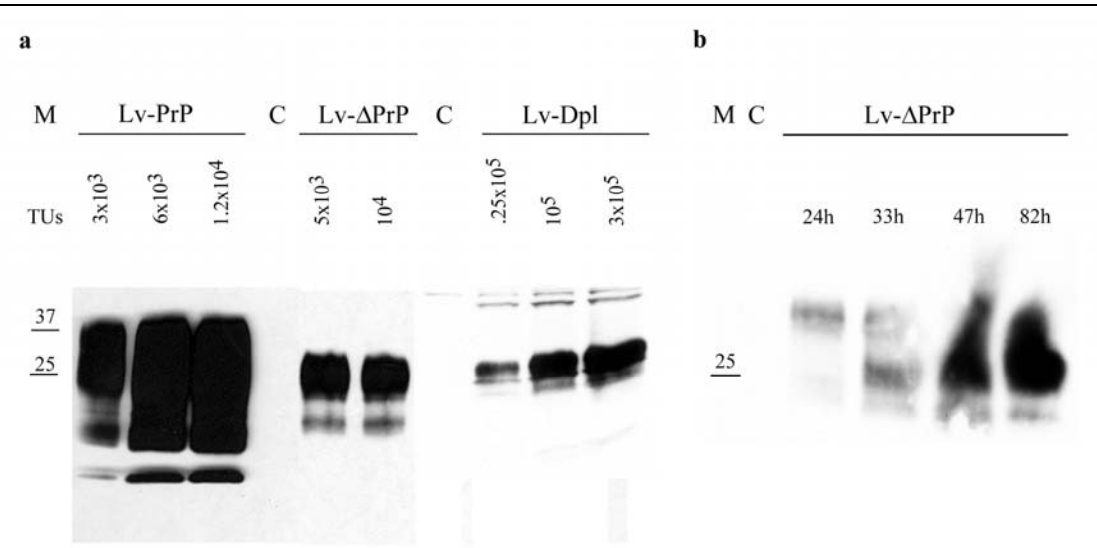


Figure 23. (a) Western blot analysis of protein expression *in* $Prnp^{o/o}$ CGCs transduced with different lentiviral constructs. Different concentrations of PrP, ΔPrP and Dpl virus were used. **(b) Time course of protein expression in $Prnp^{o/o}$ CGCs.** Faint expression can be seen 33h after transduction. MW of ΔPrP is 25kDa. C untransduced $Prnp^{o/o}$ CGCs. M Marker.

By immunocytochemistry, we evaluated if the proteins are expressed and compared the expression pattern to the one of PrP in wild-type CGCs (Fig. 24). On confocal microscopy, the expression of proteins of lentiviral-transferred cDNA for PrP, ΔPrP and Dpl resembled the expression pattern of PrP from CGCs isolated from wild-type mice. GFP expression, due to the IRES.GFP fused to each cDNA, of the transduced cells co-localized with the expression of PrP, ΔPrP and Dpl.

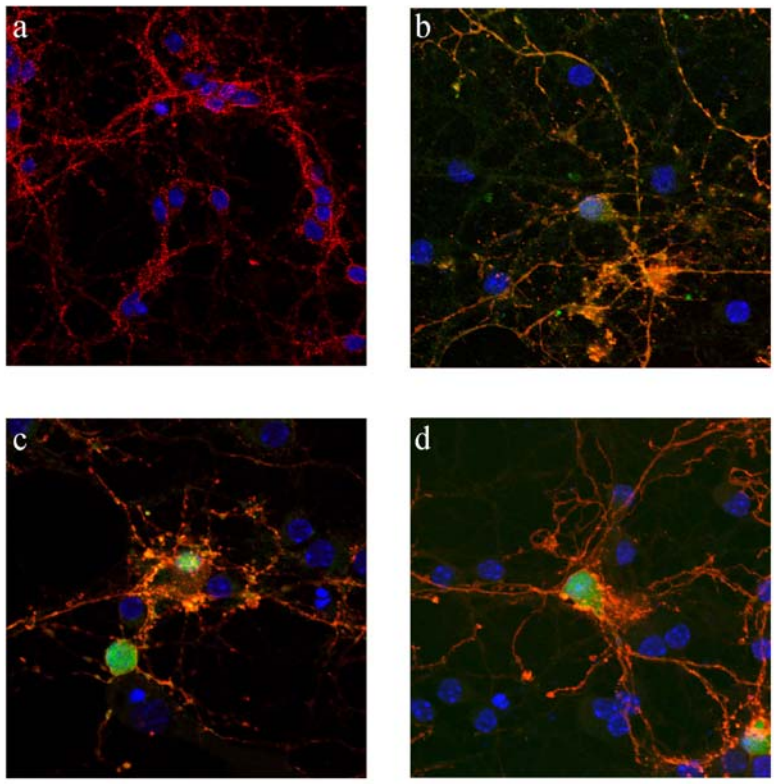


Figure 24. Immunostainings of wt CGCs and lentiviral transduced *Prnp*$^{o/o}$ CGCs. (a) CGCs from wild-type mouse. CGCs from *Prnp*$^{o/o}$ mouse transduced with lentiviral vector expressing PrP (b), ΔPrP (c) and Dpl (d). The labeling in (a), (b), and (c) was performed with monoclonal anti-PrP antibody (6H4) detected by 2nd antibody labeled with Alexa546. In (d) polyclonal anti-Dpl antibody detected by 2nd antibody labeled with Alexa546 was used. The overlapping yellow staining is indicative of PrP and eGFP co-localization. Magnification: 630x.

Transduction efficiency

We evaluated then the transduction efficiency of CGCs of the different lentiviral stocks that were independently prepared. 320'000 CGCs were seeded prior to transduction with either 2µl or 10µl of each viral stock. High transduction efficiency was obtained in the case of the CMV.eGFP virus: 40% for 2µl and 57% for 10µl (Fig.

25). In the case of PrP, ΔPrP and Dpl, the eGFP positive cells were between 4 and 10%, when the low concentration of virus was used. The higher concentration of virus led to 11-15% of eGFP positive cells.

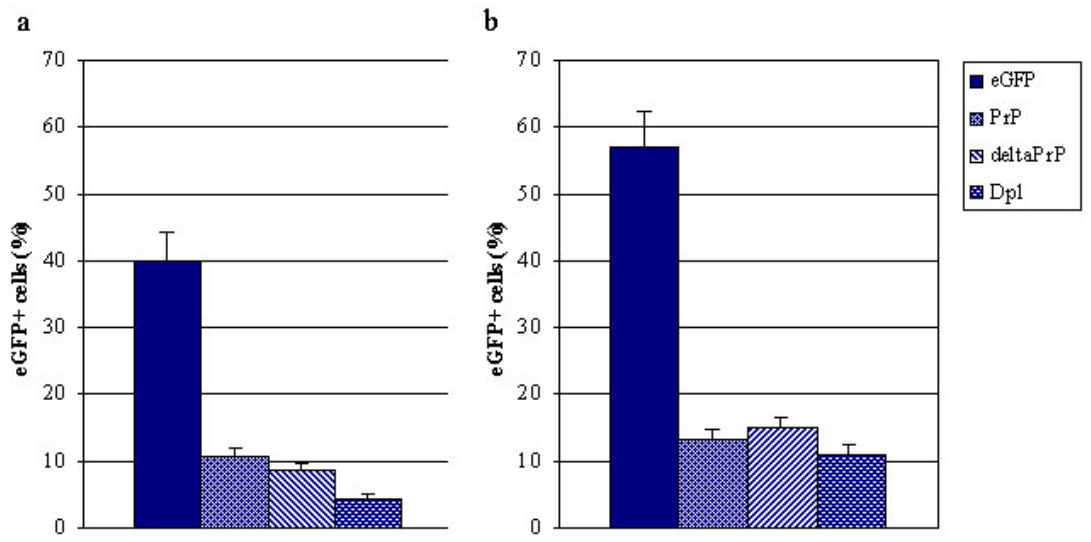


Figure 25. Efficiency of lentiviral transduction of CGCs. (a) 2µl of viral stock corresponding to $2*10^4$ TUs and (b) 10µl of viral stock corresponding to 10^5 TUs were used to transduce CGCs isolated from *Prnp*$^{o/o}$ mice.

Absence of cell death in CGCs transduced with lentivirus expressing ΔPrP and Dpl

Primary cultures of CGCs from ΔPrP^o mice did not show any sign of cell death after three weeks in culture (personal communication by Marcel Leist), which represents the upper limit of viability in general of CGCs in culture (Ishitani et al., 1996). Therefore, we thought to establish an *in vitro* model using CGCs and lentivirus to study the mechanism of ΔPrP and Dpl induced cell death observed in mice expressing ΔPrP and Dpl. CMV driven expression might lead to higher expression levels of the proteins and accelerate cerebellar granule cell death *in vitro*.

The viability of CGCs was evaluated 8 days after transduction with the three different lentiviral constructs PrP, ΔPrP and Dpl either by PI staining or by Tunnel staining. None revealed increased cell death in cells transduced with ΔPrP and Dpl lentiviral vectors compared to PrP transduced cells (Fig. 26). However, the lentivirus itself seems to have some neurotoxic effects as suggested by the significant p-values ($p<0.005$) when untransduced and eGFP transduced cells were compared. Recently,

eGFP expression has been shown to induce apoptosis in non- and neuronal cells (Detrait et al., 2002; Liu et al., 1999). But we can exclude this as a reason for the observed increased cell death in our cultures, since we did not observe a reduction of eGFP positive cells overtime. Additionally, there is also a statistically significant toxicity induced by the PrP lentivirus, and probably also by ΔPrP and Dpl, when compared to the eGFP lentivirus. Either overexpression of PrP is unexpectedly toxic and as toxic as ΔPrP and Dpl to CGCs, or the lentiviral stocks contain some contaminants, being toxic for CGCs. However, the first reason seems unlikely, as *tga*20 mice that overexpress PrP do not show any cerebellar neurodegeneration (Fischer et al., 1996). To exclude the second reason, bulk assays should be performed to verify that excess non-infectious particles and other contaminants do not interfere with vector performance (De Palma and Naldini, 2002).

In this experiment, we simply counted the PI positive cells without taking into account the eGFP positive cells, i.e. the transduced cells. However, the fields that were counted did not show any cells that were double-labeled for eGFP and PI that would indicate specific cell death in transduced cells. To analyze cell death induced by the expression of ΔPrP and Dpl in more detail, FACS analysis should be performed on eGFP positive CGCs co-stained with PI or Annexin-V as well as cytotoxicity assays and/or viability assays.

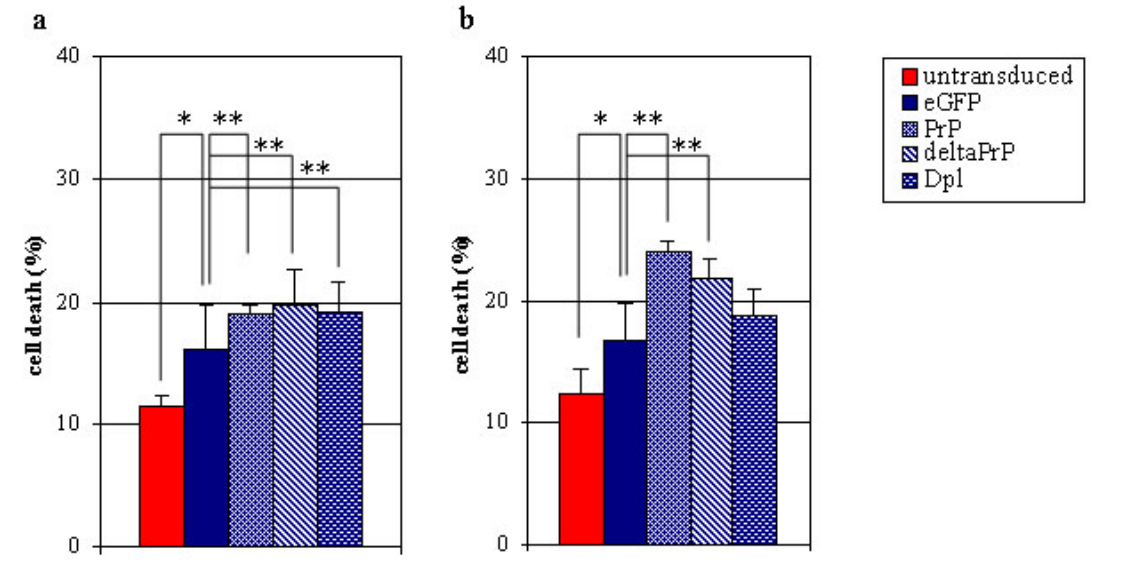


Figure 26. No cell death is induced in CGCs by lentiviral construct expressing ΔPrP and Dpl compared to CGCs transduced with PrP lentivirus. (a) 2µl of viral stock corresponding to $2*10^4$ TU and (b) 10µl of viral stock corresponding to 10^5 TU were used to transduce CGCs isolated from *Prnp*$^{o/o}$ mice. Cell death was assessed by PI staining of CGCs 8 days after lentiviral transduction. Significant differences were determined using the t test and are indicated by * with $p<0.005$, by ** with $p<0.05$ (a) and $p< 0.001$ (b).

DISCUSSION

Overexpression of Dpl in *Prnp*$^{o/o}$ mice, due to enhanced intergenic splicing when the splice acceptor site is deleted on *Prnp* exon 3, leads to progressive ataxia and cerebellar cell degeneration (Moore et al., 2001; Moore et al., 1999; Rossi et al., 2001; Sakaguchi et al., 1996). A similar phenotype was described in mice expressing the globular domain of PrP lacking the flexible N-terminus (ΔPrP) (Shmerling et al., 1998). In both cases, reintroduction of a PrP gene abrogated the cerebellar syndrome (Flechsig et al., 2003; Nishida et al., 1999; Rossi et al., 2001; Shmerling et al., 1998). Because the overall structure of Dpl is remarkably similar to that of ΔPrP, and because full length PrP antagonized both Dpl and ΔPrP induced neuronal cell death, the mechanism of pathogenesis might be the same in both ataxic syndromes (Moore et al., 1999; Weissmann and Aguzzi, 1999). In order to study this common molecular mechanism(s), we established an *in vitro* model using primary cerebellar granule neurons isolated from *Prnp*$^{o/o}$ mice and lentiviral vectors to transfer the genes of interest (*Prnd*, *Prnp*, Δ*Prnp*) to the cells. Primary cerebellar granule neurons are a well characterized model system for investigating neuronal death (Galli et al., 1995). Since they are non-dividing cells, lentiviral vectors are an adequate tool for gene transfer into neurons.

The cDNA of PrP, Dpl and ΔPrP were efficiently transferred to the *Prnp*$^{o/o}$ CGCs by the lentiviral vectors and protein expression was observed at 33h post-transduction. The expression patterns of the different proteins, expressed in CGCs of mice deficient for PrP, were comparable to the one of wild-type neurons. The number of eGFP positive CGCs between the different lentiviral stocks varied significantly, suggesting that the transduction efficiency is not yet optimally assayed. In addition to the end-point titration a bulk assay should be performed, i.e. by measuring the maximal frequency of transduction that is obtained when high vector input is used. Moreover, bulk assays verify that excess of non-infectious particles and other contaminants do not interfere with vector performance (De Palma and Naldini, 2002). To increase transduction efficiency, higher TU/ml and fewer medium will be used, so that the possibility that vectors and cells encounter each other is enhanced. Other strategies to increase the chance of vector particles to come into contact with the target cells, such

as prolonged centrifugation of vector together with the cells (spinoculation) (O'Doherty et al., 2000) will also be employed.

Surprisingly, we did not observe cell death induced by the expression of ΔPrP and Dpl when compared to PrP transduced CGCs.

This could be due to insufficient expression of the proteins to induce cell death. Cerebellar cell degeneration in ΔPrP and Dpl expressing mice has clearly been shown to be dose-dependent (Anderson et al., 2004; Rossi et al., 2001). In cells transduced with lentiviral vectors, the average expression level depends on the copy number of integrated vectors (De Palma and Naldini, 2002). Since we have used low concentrations of virus, only a few cells might have integrated multiple copies of the transgene that could lead to expression levels inducing cell death.

In addition, the absence of cell death in ΔPrP and Dpl transduced CGCs suggests that the mechanisms leading to cell death might be dependent on other cell types, e.g. Purkinje cells. It has been shown that Purkinje neurons provide critical trophic support to cerebellar granule cells (Linseman et al., 2002). Moreover, degeneration and loss of Purkinje neurons in Lurcher cerebellum (point mutation in the δ2 glutamate receptor (GluRδ2)) is followed by secondary death of cerebellar granule neurons and inferior olivary neurons attributable to loss of trophic support normally provided by their afferent target Purkinje neurons (Wetts and Herrup, 1982). However, Dpl expressed in Purkinje cells leads to Purkinje cell death in absence of secondary cerebellar granule cell death (Moore et al., 1999). Moreover, ubiquitous expression of Dpl in the brain of *Prnp*$^{o/o}$ mice causes severe ataxia associated with loss of both granule and Purkinje cells (Moore et al., 2001). In addition, overexpression of N-terminally truncated PrP targeted specifically to Purkinje cells of ZrchI PrP knock out mice (under the control of the Purkinje cell-specific L7 promoter) resulted in the same phenotype observed in the Nagasaki-type PrP knock out mice, namely ataxia and Purkinje cell degeneration (Flechsig et al., 2003). Moreover, no secondary granule cell death was reported. However, *Prnp*$^{o/o}$ mice that express Dpl under the transcriptional control of L7 promoter evidenced initial Purkinje cell death followed by granule cell death, likely as a consequence of the absence of critical inputs from Purkinje cells rather than of a direct effect of the transgene (Anderson et al., 2004). These results suggest that cell specific expression of Dpl and ΔPrP induces pathways that lead to death of the

particular cell type. However, the presence of other cell types, such as microglia and astrocytes, might be important for inducing cell death.

Another possibility is that the conditions in the culture model do not simulate the conditions leading to cell death in ΔPrP and Dpl overexpressing mice. First, primary neurons are prepared from the developing nervous system. Thus, CGCs lack the natural environment when they differentiate *in vitro* that might lead to absence of protein expression occurring *in vivo*. Second, in ΔPrP^{o} mice cerebellar granule cell apoptosis starts to be evident at the age of three weeks (Shmerling et al., 1998). However under typical culture conditions, cerebellar granule cells die after about 17 days in vitro (Ishitani et al., 1996).

In addition, CGCs require activity-dependent signals (membrane depolarization) and serum for their survival *in vitro* (D'Mello et al., 1993; Galli et al., 1995; Miller et al., 1997). After removal of serum and lowering of extracellular potassium form 25 to 5mM, CGCs undergo rapid apoptotic cell death characterized by caspase activation and nuclear condensation and fragmentation. Therefore, triggering cell death in lentiviral vector-transduced cultures by withdrawal of serum and potassium might reveal a difference in survival between PrP, ΔPrP and Dpl transduced neurons. Introduction of one transgene of full-length PrP in the Dpl or ΔPrP overexpressing mice protects the neurons from dying, suggesting a neuroprotective role of PrP^{C}. The neuroprotective function of PrP seems to require the octapeptide repeats, because the deletion of amino acids 23-88 abrogates the ability of PrP (hamster/mouse chimeric PrP) to protect against Dpl (Atarashi et al., 2003). In contrast, mice expressing ΔPrP crossed with mice expressing PrP lacking the octarepeats (PrPΔ32-93) completely suppress the neurological syndromes. However, PrPΔ32-106 could no longer abolish the defect (Flechsig and Weissmann, 2004). It has also been shown that the absence of PrP *in vitro* or *in vivo* contributes to an increased susceptibility to oxidative stress (Brown and Besinger, 1998; Brown et al., 2002) or apoptosis-inducing insults (Chen et al., 2003; Mouillet-Richard et al., 2000). These data suggest that PrP exerts a neuroprotective function (for review (Roucou et al., 2004)). In contrast, ΔPrP and Dpl has been shown to induce neurodegeneration *in vivo* as well as *in vitro* (Anderson et al., 2004; Cui et al., 2003; Daniels et al., 2001; Flechsig et al., 2003; Moore et al., 2001; Rossi et al., 2001; Shmerling et al., 1998)

In order to identify the mechanism(s) responsible for cell death in the cerebellum in mice expressing Dpl and ΔPrP, we intend to perform microarrays on CGCs transduced with the various lentiviral vectors. Cell death might not be evident in these cultures due to the limited lifespan of CGCs in culture, but subtle changes at the molecular level might already be detectable in Dpl and ΔPrP compared to PrP transduced cerebellar granule cells.

Moreover, in order to model more adequately an *in vivo* situation, we plan to use cerebellar slices and to transduce the cells with the different lentiviral vectors. This model might enable us to evaluate the importance of the presence of other cell types for the induction of cerebellar granule cell death.

We might also test different conditions, e.g. lowering potassium levels and serum concentration, in order to study the susceptibility of CGCs to cell death, that are transduced with the various lentivirus. Proteasome inhibitors added to cultures might also imbalance the cell culture system toward cell death, when cells are transduced with Dpl and ΔPrP but not when transduced with PrP.

Recently, it has been proposed that mutant prion pathology was derived from altered processing of misfolded prion and retrograde transport to the cytosol (Ma and Lindquist, 2001; Ma et al., 2002). Inhibition of proteasome function in neuronal cell lines highly expressing PrP^C as well as expression of a PrP mutant targeted to and accumulating in the cytosol (cyPrP), induced neuronal death in vitro and neurodegeneration in transgenic mice (Ma et al., 2002). Does ΔPrP induce cerebellar granule cell death by a similar mechanism? However, several observations argue against a similar mechanism of neurotoxicity for ΔPrP. First, ΔPrP retains the secretory signal sequence and the GPI anchor site needed for appropriate targeting to the secretory pathway and for attachment for the plasmalemma. Second, ΔPrP pathology is suppressed by co-expression of wild-type PrP^C, whereas cyPrP induces pathology regardless of PrP^C expression.

Cross-linking of cellular prion protein by specific monoclonal antibodies has been shown to trigger extensive apoptosis in hippocampal and cerebellar neurons (Solforosi et al., 2004). Our in vitro model might be suitable to test a variety of antibodies for their capability to induce cell death and to map the regions of PrP accessible to cross-linking. The mechanisms of cell death could then also be analyzed in order to clarify if cell death is induced by a loss of function of PrP, such as mediating a survival

signal, or by initiating an apoptotic cascade. Moreover, Solforosi *et al*. (Solforosi et al., 2004) suggested that in prion-infected brains, neuronal loss may occur when oligomeric forms of cell surface PrP^{Sc} (Lehmann and Harris, 1996; Vey et al., 1996) undertake the PrP^{C} cross-linking role.

With this in vitro model, we have a powerful tool to analyze death or survival pathways and, hopefully, to elucidate the physiological function of the cellular prion protein.

PART II

Efficient prion inactivation, solubilization and facilitation of LC-MS/MS analysis of PrP^{Sc} by the anionic detergent *sodium 3-[(2-methyl-2-undecyl-1, 3-dioxolan-4-yl) methoxyl]-1-propanesulfonate* (MPS).

INTRODUCTION

Protein aggregation and neurotoxicity

Abnormal protein aggregation is a key feature of a number of other neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's diseases and amyelotrophic lateral sclerosis (Aguzzi and Haass, 2003; Murphy, 2002; Selkoe, 2003; Taylor et al., 2002b). These protein aggregates share several physicochemical features: a fibrillar morphology, a predominantly beta-sheet secondary structure, birefringence upon staining with the dye Congo red, insolubility in common solvents and detergents, and protease-resistance. None of the polypeptides implicated in these diseases exhibit any primary sequence homology, nor do they derive from similar sources. However, non-conservative gene mutations have been identified for each of these disorders, contributing to protein misfolding, aggregation and eventual deposition in neuronal inclusions and plaques. These diseases lead to extensive degeneration of neurons. In Huntington's disease, neuronal loss is believed to be directly mediated through the aggregated state of the huntingtin protein (Bates, 2003; Meriin et al., 2002). In other cases (for example the beta-amyloid and alpha-synuclein proteins in Alzheimer's and Parkinson's disease respectively), presumptive neurotoxic oligomeric protein-intermediates, referred to as protofibrils, have been suggested to mediate cell death (Lashuel et al., 2002). In prion diseases, the precise role of misfolded, aggregated scrapie prion protein in the neurodegenerative process remains controversial. The protein-only prion hypothesis of protein replication, initially conceived in 1967 (Griffith, 1967) and subsequently refined by Stanley Prusiner (Prusiner, 1982) states that the prion (of which PrP^{Sc} constitutes the major, if not sole

component) is the infectious and neurotoxic agent. However, work by Brandner *et al.* (Brandner et al., 1996a) and recently by Mallucci and coworkers (Mallucci et al., 2003) provide evidence *in vivo* that accumulation of PrP^{Sc} in the brain per se does not cause neurodegeneration. Rather, this neurotoxicity may be mediated by the conversion of the cellular isoform (PrP^{C}) to disease-associated isoforms (PrP^{Sc}) specifically within or on neurons expressing the prion protein. Or, because PrP^{Sc} binds PrP^{C} (Horiuchi and Caughey, 1999b), it may cause a loss or alteration of the physiological function of PrP^{C}. However, postnatally induced *Prnp* ablation does not elicit any phenotype (Mallucci et al., 2002), hence prion pathology is unlikely to come about by a loss of PrP^{C} function.

A general mechanism for neurotoxicity in all neurodegenerative diseases based on protein aggregation has recently been suggested (Bucciantini et al., 2002; Kayed et al., 2003). Bucciantini and colleagues were able to demonstrate that aggregation of non-disease associated proteins exhibit inherent cytotoxicity. Moreover, it has recently been demonstrated that different types of soluble amyloid oligomers potentially share a common structure that is recognized by a single antibody irrespective of the primary amino acid sequence (Kayed et al., 2003). The binding of this antibody neutralizes the cytotoxic effects of these oligomers, again suggesting a common mechanism of toxicity for all these oligomers. However, despite this recent progress, understanding of the precise pathophysiological mechanisms that eventually lead to the death of neurons is currently lacking.

Proteomic analysis in neuroscience

Recent advances in instrument technology, bioinformatics and molecular biology (genomics, proteomics, functional genomics) are making it possible to simultaneously analyze entire complements of genes expressed in particular cells or tissues. Complementary insights into biological or disease related processes have been obtained by proteomic studies. The field of neuroscience is particularly well suited to analysis with these new techniques, given the complexity of neuronal signaling and the diversity of cellular responses.

The concept of the proteome was recently defined as “The entire protein complement expressed by a genome or by a cell or tissue type” (Wilkins et al., 1996). A two-step

process generally drives proteome analysis: protein sample preparation (i.e. purification, separation) and the subsequent analysis by mass spectrometry (MS). The broadest proteome coverage is probably obtained from a combination of multidimensional fractionation and advanced MS instrumentation. Multidimensional separations are a prerequisite for the detection of lower abundant proteins. Several protein fractionation methods have been developed such as two-dimensional polyacrylamide gel electrophoresis 2D-PAGE (Hanash, 2001) or multidimensional protein identification technology (MudPIT) (Washburn et al., 2001) and applied to research in neurodegenerative diseases (Butterfield and Castegna, 2003; Zou et al., 2003). Multidimensional chromatography in conjunction with isotopic labeling allows to identify and at the same time quantify proteins in complex mixture (Gygi et al., 1999; Han et al., 2001; Ideker et al., 2001; von Haller et al., 2001). The prototypical method to generate quantitative protein profiles based on stable isotope affinity tagging and MS is the isotope-coded affinity tag (ICAT) reagent method (Gygi et al., 1999). This method makes use of the facts that pairs of chemically identical analytes of different stable-isotope composition can be differentiated in a mass spectrometer owing to their mass difference, and that the ratio to signal intensities for such analyte pairs accurately indicates the abundance ratio for the two analytes. A typical experiment compares the protein expression levels of two samples. After appropriate purification each fraction is tagged with either of the two available ICAT reagents that specifically label cysteine residues ($^{13}C_0/^{13}C_9$). After terminating the labeling reaction, the two samples are pooled and tryptically digested. The pooling prevents an alteration of the protein ratios that may occur due to uneven sample handling. Depending on sample complexity, the resulting peptide mixture is fractionated by multi-dimensional chromatography and purified through avidin affinity columns. Finally, labeled peptides are analyzed using tandem mass spectrometry. Ad-hoc developed software (i.e. SEQUEST, INTERACT, XPRESS) (Han et al., 2001) allows automated identification, quantification, and data mining of every peptide (Fig. 27).

The ICAT technology is also well suited to analyze protein interaction studies. In such experiments, accurate quantification by means of stable-isotope labeling is not used for protein quantification per se; instead the stable isotope ratios distinguish between the protein compositions of two or more protein complexes. It would therefore be well suited to compare prion-infected brain to non-infected brain, and subsequently, to identify components of protein aggregates and to assess whether these represent

factors that are involved in PrP^{Sc} conversion and aggregation or play a role in the disease-related neurodegenerative processes. In conjunction with mass spectrometry, this technology allows for detection of low abundant proteins. Therefore it might be suitable for establishing a diagnostic test that allows screening for low prion titer in blood or urine. The currently available diagnostic tests for prion diseases can only be made by analysis of biopsy or autopsy material, thereby detecting protease resistant PrP^{Sc} or PrP^{27-30} (the protease resistant core after PK digestion) in tissue samples by Western blot analysis or immunohistochemistry.

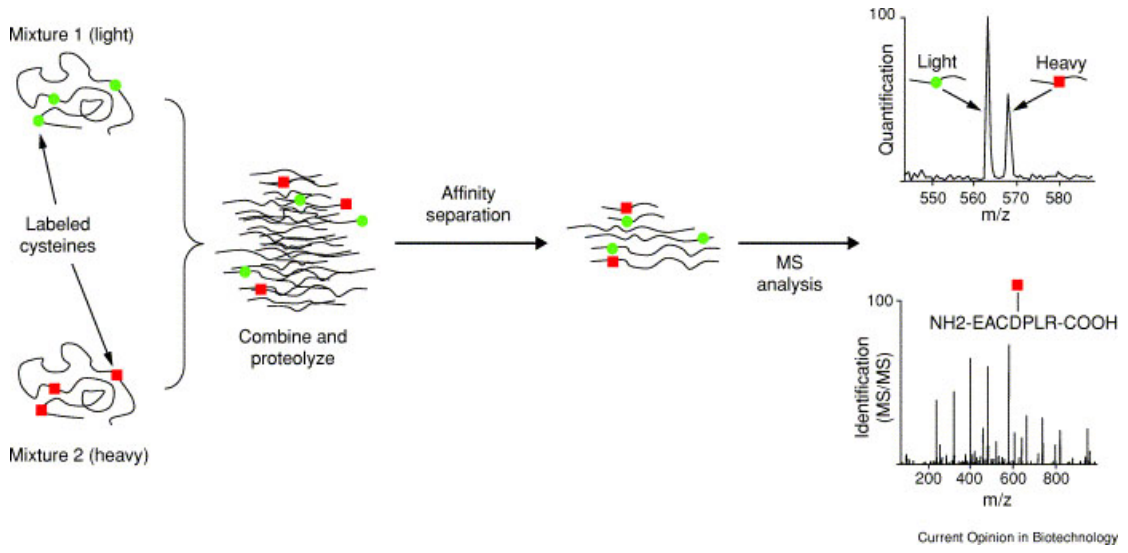


Figure 27. The ICAT reagent strategy for quantifying differential proteins. Two protein mixtures representing tow different cell states are treated with the isotopically light (green) or heavy (red) ICAT reagents, respectively. The labeled protein mixtures are then combined and proteolyzed; tagged peptides are selectively isolated, analyzed by MS, quantified and identified (Tao and Aebersold, 2003).

Limitation and drawbacks of proteomics of neurodegenerative diseases

However, the application of powerful proteomic technologies on neurodegenerative disease research is often impaired by the insoluble nature of the disease-associated protein aggregates. Harsh conditions, *e.g.* 80% formic acid treatment of A-beta amyloid plaques (Harigaya Y. et al., 1995) and the exposure of PrP^{Sc} to Gdn-HCl or urea (Prusiner et al., 1993; Prusiner et al., 1981), are required to denature and solubilize the proteins. In the case of prion diseases these steps are frequently required to inactivate the infectious agent prior to sample processing and analysis out of BSL3 containment facilities. Perhaps more significantly, these conditions are incompatible

with subsequent sample fractionation and processing. In particular, liquid chromatography tandem mass spectrometry (LC-MS/MS) based analyses require specific proteolytic cleavage by endopeptidases such as trypsin. However, even slightly unfavorable conditions will either inhibit protein cleavage, or favor non-specific cleavage events, hampering MS analysis. Thus, the mentioned solubilizing agents for protein aggregates are required to be neutralized, significantly diluted, or fully removed by time-consuming additional procedures prior to further biochemical processing and analyses.

Outline of this work

To enable and facilitate our mass spectroscopic studies of PrP^{Sc} aggregates in prion disease, we sought to establish procedures that would allow us to analyze protein aggregates and associated factors in a reproducible manner using ICAT labeling and LC-MS/MS. Based on experiments with aggregated PrP^{Sc}, we have tested different denaturing agents to solubilize aggregated proteins and finally developed a method using the novel surfactant *sodium 4-[2-methyl-2-undecyl-1, 3-dioxolan-4-yl) methoxyl]-1-propane sulfonate* (RapiGest™, Waters; in this study referred to as MPS). MPS is a mild denaturant that facilitates solubilization and unfolding of proteins, especially hydrophobic proteins/peptides, rendering them more amenable to cleavage without inhibition of commonly used proteases. The reagent is acid sensitive, thereby allowing for easy removal from the analytes, and does not interfere with downstream post preparation methods such as HPLC or LC-MS/MS. We demonstrate that MPS allows for solubilization of inherently insoluble PrP^{Sc} and allows it to be accessible for proteomic analysis by mass spectrometry. Additionally, MPS renders partially proteinase K resistant PrP^{Sc} PK-sensitive and reduces prion-infectivity titers by several orders of magnitude (approximately 6 logs LD50).

RESULTS

GdnSCN and urea are incompatible with trypsin digestion

We wished to establish a simple protocol that is compatible with currently available quantitative proteomics technologies, while addressing the processing of samples that contain highly insoluble protein fractions. We therefore assessed the use of a range of reagents for their efficacy in solubilization of aggregated prion protein PrP^{Sc}, thereby conferring accessibility for further proteolytic cleavage.

For this purpose, 10% brain homogenate of prion infected CD1 mice at terminal stage of disease was processed and utilized in a number of ways: Direct exposure of the PK treated material to trypsin did not allow cleavage of the PK resistant core of PrP^{Sc} as assessed by Western blot analysis (Fig. 28a). Next we tested whether GdnSCN or urea, both known to disaggregate PrP^{Sc} at least partially (Prusiner et al., 1993), were capable of rendering cleavage sites accessible to trypsin. As shown in figure 28b, trypsin digestion of PK digested PrP^{Sc} was incomplete when treated either with 8M urea for 2h and 24h or with 6M GdnSCN for 24h. Longer incubation in 8M urea at room temperature was even less favorable than 2 hours incubation and resulted in reduced tryptic cleavage of PrP^{Sc}. This may be due to carbamylation of lysine and arginine residues (McCarthy et al., 2003). In this process, urea decomposes to isocyanic acid that subsequently reacts with the amino terminus of peptides or with the side chains of lysine and arginine residues and results in inhibition of proteolysis by trypsin. As strong denaturing conditions (i.e. 6M GdnSCN or 8M urea, respectively) were not compatible with the subsequent proteolytic cleavage step, GdnSCN treated samples had to be dialyzed against H_20 prior to cleavage, and the urea concentration had to be adjusted to 1M in the urea-treated samples. To circumvent these problems, an agent that would solubilize and unfold proteins, without having the incommoding effect of denaturing or inhibiting common proteolytic enzymes would be highly desirable.

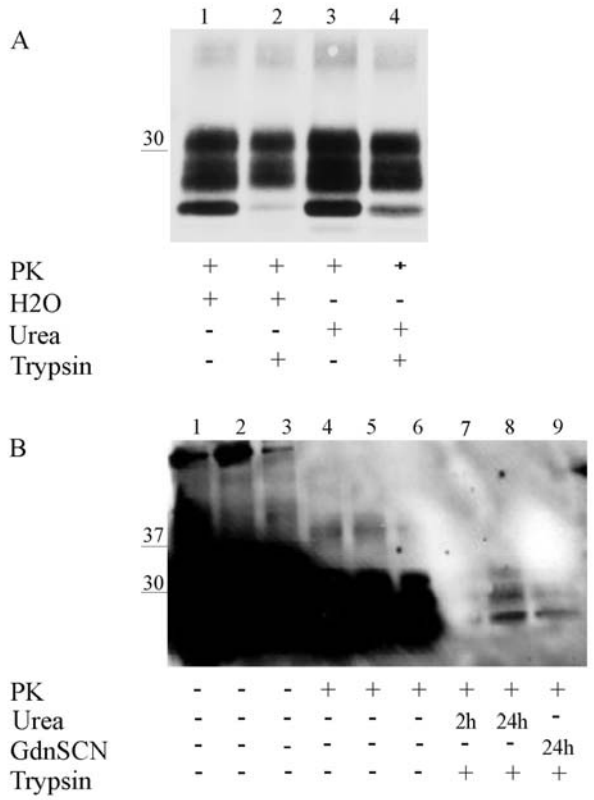


Figure 28. Urea and GdnSCN are incompatible with trypsin digestion. A PrP^{Sc} after PK treatment, resist to trypsin digestion (lane 2 and 4) independently of PrP^{Sc} exposure at RT for 2h to H20 (lane 1 and 2) or 8M urea (lane 3 and 4). For trypsin digestion, sample containing 8M urea was diluted to 1M urea to ensure efficient tryptic digestion. **B** PrP^{Sc} were exposed to either 8M urea for 2h or 24h (lane 7 and 8) or 6M GdnSCN for 24h at RT (lane 9). After dilution to 1M urea respectively dialysis against H2O, samples were digested with trypsin O/N at 37°C. Lane 1, 2 and 3: PrP^{Sc} samples prior to exposure to PK, detergents and trypsin. Lane 4, 5 and 6: PrP^{Sc} samples after PK digestion. Longtime exposure of membrane to film.

A novel surfactant "sodium 4-[2-methyl-2-undecyl-1, 3-dioxolan-4-yl) methoxyl]-1-propane sulfonate"(MPS) efficiently solubilizes PrP^{Sc}

MPS, a newly available detergent, was specifically developed to render hydrophobic proteins amenable to enzymatic digestion. It is compatible with all common used proteases, such as Lys-C, Asp-N and Glu-C endoproteinases, and chymotrypsin. Trypsin digestion was shown to be greatly enhanced and accelerated even at MPS concentrations of up to 2% (see product description of MPS).

In order to determine to what extent MPS allowed solubilization of PrP^{Sc}, PK-treated samples were incubated at RT and at 95°C and exposed to 1 or 2% of detergent. Thereafter, the samples were incubated with trypsin at 37°C overnight, and the

cleavage efficiency determined by Western blotting (Fig. 29a/b). The cleavage efficiency directly correlated with detergent concentrations, incubation temperature and time. Incubation at 95°C for 1 hour at a final detergent concentration of 2% resulted in almost complete trypsinization of the PK-resistant core of PrP^{Sc}, whereas incubation at room temperature (data not shown) and low MPS concentrations were ineffective (Fig. 29a and b). PK treated PrP^{Sc}, remaining after tryptic digestion O/N at 37°C, could be completely processed by an additional incubation of 6h adding fresh trypsin (Fig. 29c, lane2). Detergent concentrations higher than 2% were not tested since they are reported to increasingly interfere with subsequent proteolytic cleavage (see product description of MPS).

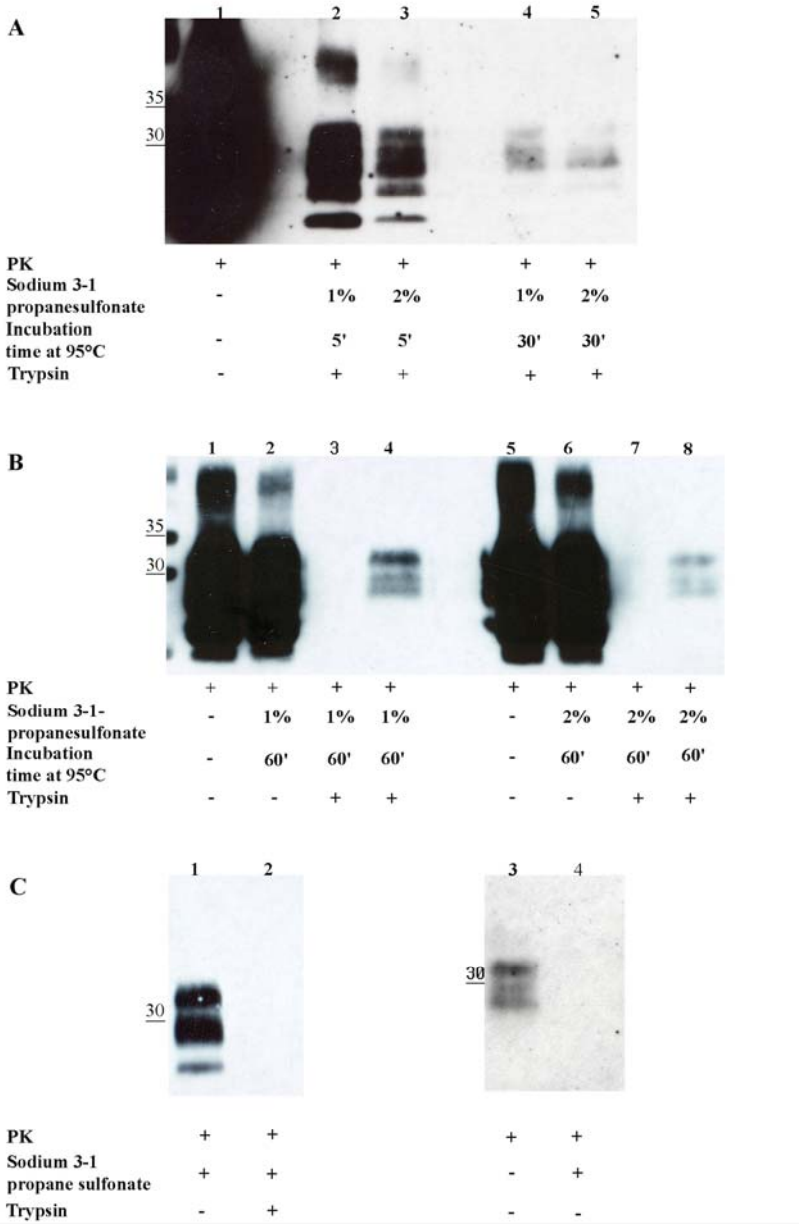
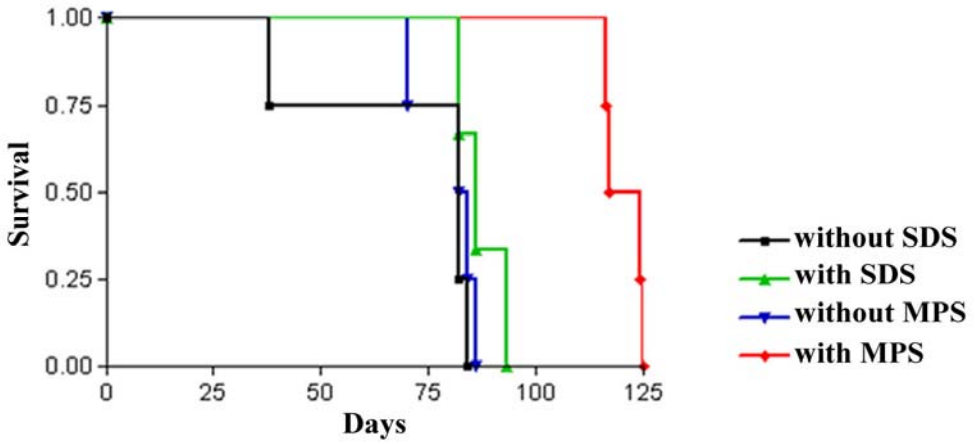


Figure 29. Concentration and time dependent solubilization effects of MPS. A PrP^{Sc} was exposed to PK and incubated in 1% (lane 2 and 4) or 2% MPS (lane 3 and 5) for 5 or 30min. Thereafter, the samples are trypsinized O/N at 37°C. **B** PrP^{Sc} was exposed to PK and incubated in 1% or 2% MPS for 60min. The samples are trypsinized subsequently O/N at 37°C. **C** lane 1: PrP^{Sc} treated with PK (control positive), Lane 2: PrP^{27-30} exposed to 2% MPS for 60min. Thereafter, it was trypsinized twice. Lane 3: PrP^{Sc} PK digested, but not exposed to MPS (control positive), lane 4: After exposure to 2% MPS for 60min, PrP^{Sc} is PK sensitive.

MPS reduces prion infectivity

When PrP^{27-30} is exposed to MPS for 1h at 95°C, PrP^{27-30} becomes sensitive to PK, whereas untreated material still displays PK resistance (Fig. 29c, lane 3 and 4). This prompted us to ask whether this PK sensitivity would correlate with a reduction of infectivity titers as it has been described for SDS, GdnSCN and urea (Prusiner et al., 1993; Taylor et al., 1999). To assess whether MPS is able to inactivate prions, we performed a mouse bioassay using *tga20* mice that overexpress murine PrP^{C} (Fischer et al., 1996). One group of mice was inoculated with 30µl of 0.75 x 10^{6} RML 5 that had been exposed to 2% MPS at 95°C for 1h. 0.1% SDS as well as PK, that was preliminary heat-inactivated, were also present in order to simulate the conditions that are used for sample preparation for mass spectrometry analysis (see method and material). For control, an age-matched group of mice was inoculated with RML 5 that had been digested with PK (50µg/ml, for 30 min at 37°C) in the presence of 0.1% SDS. This amount of SDS enhances the PK activity and allows for a shorter incubation time of PrP^{Sc} in order to become PrP^{27-30}. The control group was to estimate the reduction of the titer by exposing PrP^{Sc} to this low amount of SDS and PK treatment. As depicted in figure, the low amount of 0.1% SDS in conjunction with PK digest can reduce the titer of prion infectivity by approximately 3 logs. Even more important, confirming our solubilization results, 2% MPS in the presence of 0.05% SDS is able to reduce infectivity titers by 5-6 logs LD50 (Fig. 30).

A



B

Inoculum	Attack rate	Incubation time in days ± SE	Infectivity (log LD 50/ml)	Reduction factor
Sample **not** treated with **SDS** 1:17400	3/3 *	82.7 ± 1.2	4.2	
Sample treated with **SDS** 1:40	3/3 **	89.8 ± 7.1	3.6	4.8x10^2.6 **=10^3.3**
Sample **not** treated with **MPS** 1:18200	4/4	80.5 ± 7.2	4.4	
Sample treated with **MPS** 1:80	4/4	120.5± 4.7	0.9	2.5x10^5.5 **=10^5.9**

* 1 mouse died of undefined cause and was not counted in the experiment.
** 1 mouse alive probably due to inoculation failure. It was not counted in the experiment.
y=11.45-0.088*x (x in days)

Figure 30. Infectivity bioassay. A Survival curve of mice inoculated with RML 5 which was subjected to different treatments: without/with SDS, without/with RapiGest. **B** Infectivity titers as well as reduction factor were calculated on the basis of y=11.45-0.088* x (x in days).

As the reduction of the titer is close to the detection limit of the bioassay, we thought to further reduce the infectivity titer by incubating RML 5 24 h at 95°C instead of 1h at 95°C in 2% MPS or 4% MPS. Prior to inoculation in *tga20* mice, the presence of PrP^{Sc} in the inoculation samples was checked by Western blot (Fig. 31). Surprisingly, PrP^{Sc} was no longer detectable after 12 h of incubation. We then performed an experiment to analyze if MPS could be responsible for the loss of PrP^{Sc}. Therefore, we compared RML 5 incubated in MPS to RML 5 incubated in H_2O for 12 h at 95°C. The PrP^{Sc} signal also vanished when it was exposed to H_2O for 12 h at 95°C. Was PK inactivation at 95°C for 30min insufficient and did PK therefore retain some activity that was able to degenerate PrP^{Sc}? RML 5 was exposed to H_2O alone, H_2O and heat-

inactivated PK, or H_2O, SDS and heat-inactivated PK. These three samples were then heated at 95°C for 1h and 12h. As can be seen in figure 31, incubation for 1 h at 95°C seemed not to reduce the PrP^{Sc} content, however PrP^{Sc} disappeared completely after 12h incubation at 95°C. Not only PrP^{Sc} disappeared, but also all other proteins were lost after 12h incubation at 95°C illustrated in figure by the disappearance of actin. In order to exclude that tryptic-digested PrP^{Sc} loss is due to its exposure to 37° overnight, PrP^{Sc} was incubated in MPS only at 37°. PrP^{Sc} was still fully detectable after this treatment (data not shown).

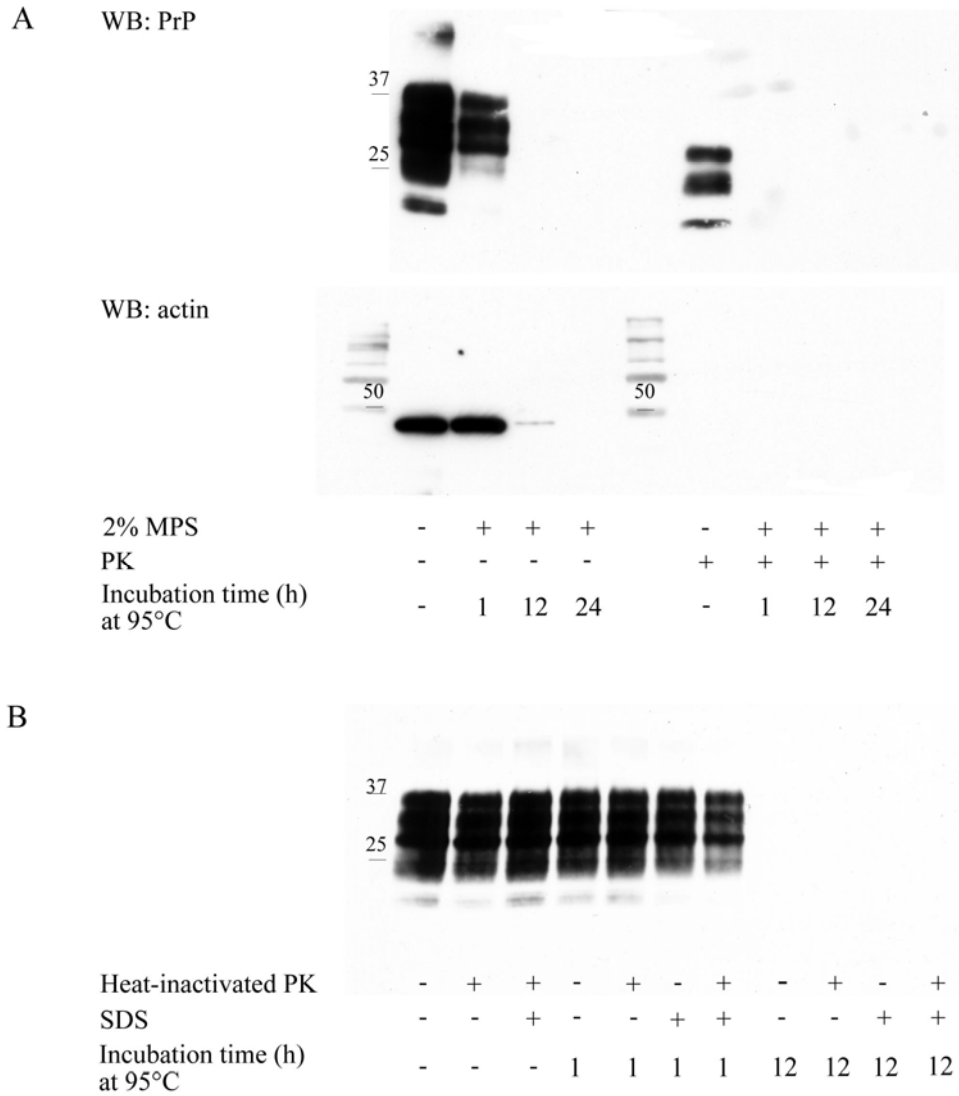


Figure 31. PrP^{Sc} signal on Western blot disappeared after 12h incubation at 95°C. A Western blot for PrP and actin after incubation in 2% MPS at 95°C for 1, 12 and 24h. **B** Western blot for PrP after incubation in H_2O for 1 and 12h at 95°C.

PrP^{Sc} tryptic peptides are readily detected by mass spectrometry following MPS treatment

To determine if tryptic peptides of PrP^{Sc} are detectable by LC-MS/MS, we first enriched for PrP^{Sc} by the sodium phosphotungstic acid precipitation (NaPTA) assay which selectively precipitates PrP^{Sc} and not PrP^{C} (Safar et al., 1998; Wadsworth et al., 2001). The resultant pellet was treated with PK to remove contaminating PK sensitive proteins that co-precipitate with PrP^{Sc}. Following PK digestion the protein pellet was resolubilized by short heating in the presence of MPS. As can be seen in figure a, following SDS-PAGE and silver staining, three bands are visible at the molecular weight of $PrP^{27\text{-}30}$, as would be expected for the proteolytic core of PrP^{Sc}. Analysis of this material by mass spectrometry revealed that we were able to detect tryptic peptides with a total coverage of approximately 60% of the amino acid sequence of $PrP^{27\text{-}30}$ (Fig. 32b).

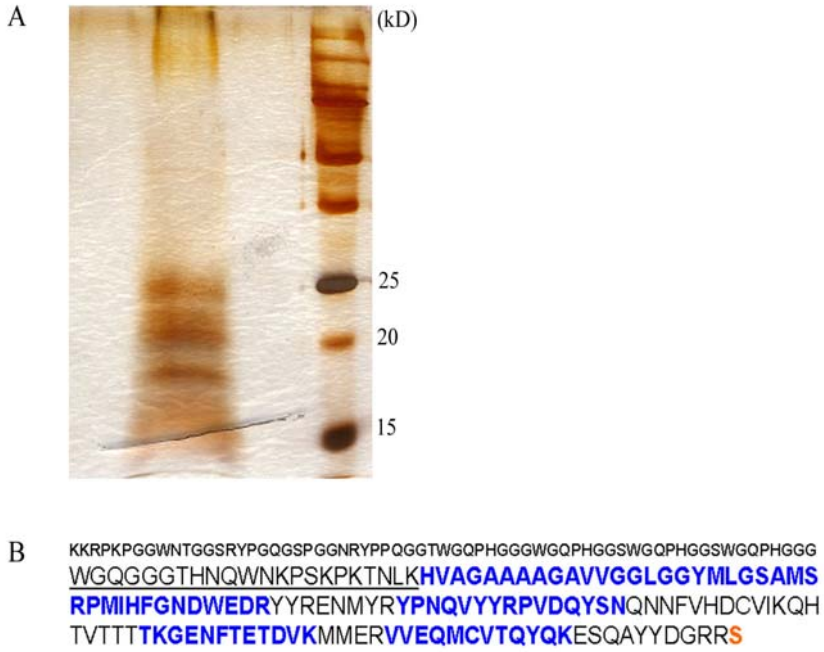


Figure 32. PrP^{Sc} peptides are detected by mass spectrometry after treatment with MPS. A Silverstaining of precipitated and PK treated infectious brain homogenate. **B** Peptides of material in A (in blue) detected by mass spectrometry cover 60% (of 84% detectable) of the amino acid sequence of $PrP^{27\text{-}30}$.

MPS is compatible with ICAT labeling

To identify components of protein aggregates and to assess whether these represent factors that are involved in PrP^{Sc} conversion and aggregation, we utilized the quantitative method based on stable-isotope labeling to distinguish between the protein compositions of two or more protein complexes. In the case of a sample, containing a protein complex, and a control sample, containing only contaminating proteins, the method can distinguish between true complex components and nonspecifically associated proteins. In addition, ICAT technology allows protein quantification that could be exploited to quantify PrP^{Sc} molecules in a given sample.
We therefore tested whether the solubilization of proteins with MPS is compatible with the ICAT technology. In the standard protocol the proteins are dissolved in a basic Tris-HCl/EDTA (pH 8.4) buffer containing 6M urea and 0.05% SDS that guarantee for an efficient protein solubilization. For the subsequent tryptic digest of the proteins, the sample has to be diluted at least 6-fold to reduce the concentration of urea to equal or less than 1M. As this step may interfere with efficient processing of aggregated proteins, we assessed whether urea could be replaced by MPS in the ICAT labeling buffer. For this purpose equal amounts of bovine serum albumin (BSA) were dissolved in labeling-buffer either containing 6M urea or 2% MPS. The proteins were solubilized at room temperature, reduced and differentially labeled with the light (sample in urea-buffer) or heavy (sample in MPS) ICAT labels. The reaction was stopped with DTT. The samples were then combined and digested with trypsin after diluting the sample to a final concentration of 1M urea. The efficiency of the labeling process and of the tryptic digest was monitored on a silverstained gel (Fig. 33a and b).

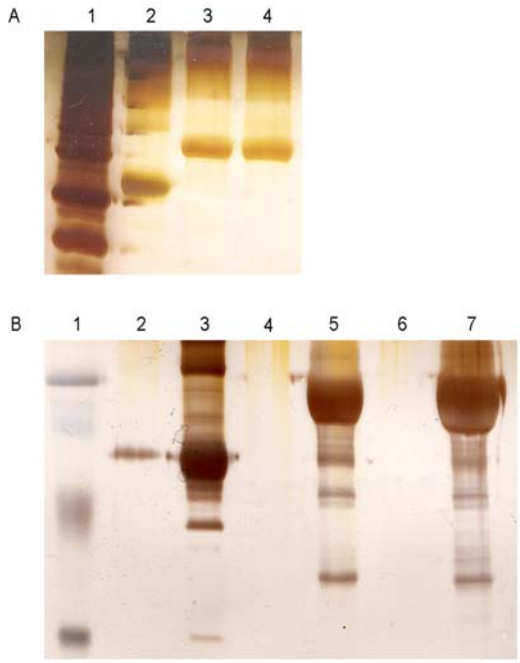


Figure 33. Silverstaining of samples digested with trypsin. A Silverstaining of BSA protein, before (lane 2) and after ICAT labeling using either a buffer containing urea (lane 3), or a buffer containing MPS (lane 4). **B** Silverstaining of BSA protein, before (lane 3) and after ICAT labeling using buffer containing MPS (lanes 5 and 7). Lane 2, 4 and 6 are empty lanes.

The digested peptides were affinity purified and prepared for LC-MS/MS analysis (for details see Materials and Methods). As depicted in figure 34 b and c, the measured set of light and heavy-labeled BSA peptides appeared with the same relative abundance (*i.e.* a ratio of 1:1) indicating that the labeling-efficiency was identical for both samples. These results were confirmed by exchanging light and heavy labels and repeating the entire procedure. Next, we replaced urea by MPS in both samples. The BSA samples were incubated either at RT or at 95°C in 2% MPS (final concentration 1.4%) for 60 minutes, differentially labeled and finally combined as described above. The tryptic digest was performed without sample dilution and the peptides were processed as described above. LC-MS/MS analysis revealed that the relative abundance of light and heavy labeled peptides was unchanged, indicating that the labeling efficiency was equal and that MPS did not interfere with mass spectrometric analysis (Fig. 34c). In addition, a slightly better sequence coverage (Fig. 34a) for BSA was obtained (with a tendency to generate fewer peptides with a missed tryptic cleavage site) when compared to the previous experiments indicating that urea can be replaced by MPS in any ICAT experiment without any constraints.

A

>SW: ALBU_BOVIN P02769 bos taurus (bovine). serum albumin precursor

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVK
LVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRETYGDMADCCEKQEPERNECF
LSHKDDSPDLPKLKPDPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQ
ECCQAEDKGACLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVE
VTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAE
VEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEA
TLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQ
VSTPTLVEVSRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKCCTESL
VNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLK
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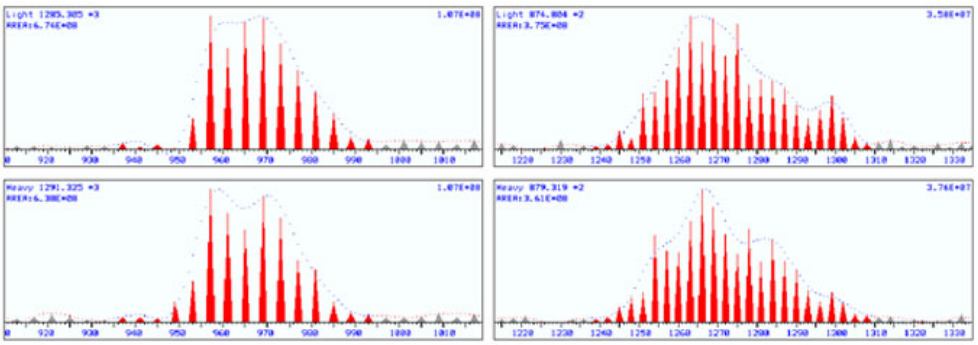
B

SHCIAEVEKDAIPENLPPLTADFAEDKDVCK LKPDPNTLCDEFK



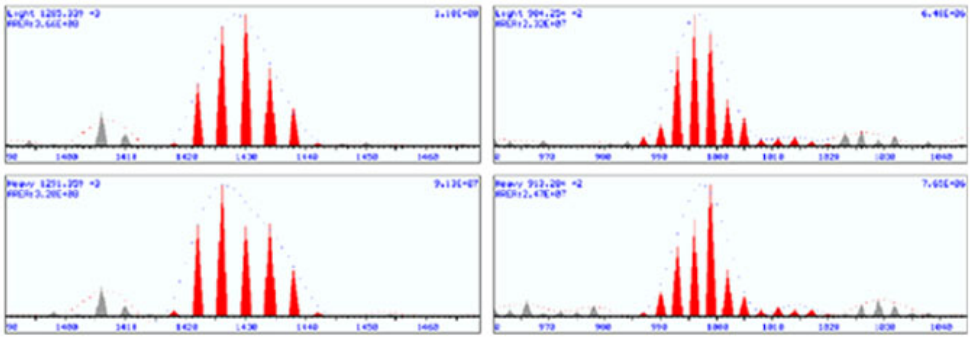
Light: Heavy
1: 1.01

Light: Heavy
1: 0.96

Urea: Rapigest

C

SHCIAEVEKDAIPENLPPLTADFAEDKDVCK LKPDPNTLCDEFK



Light: Heavy
1:0.88

Light: Heavy
1:0.83

Rapigest only

Figure 34. MPS is compatible with ICAT labeling. A Protein sequence of the BSA protein. Cystein residues are labeled in orange, identified peptides are shown in blue (a total of 19 to 21 (76-84%) out of 25 (100%) possible cystein-containing peptides were detected). Peptides identified by MPS only incubation are underlined. Missed cryptic cleavage sites are more often observed after incubations with urea in the incubation buffer. A more complete digest is obtained when incubation with MPS (data not shown). **B and C** MS-based elution profile for two representative peptides from a sample labeled in buffer containing urea or MPS (B, silverstain fig. 33 A), or MPS only (C, silverstain fig. 33 B). Note the precise co-elution of light and heavy peptides and the conserved 1:1 ratio in both experiments.

Quantitative analysis of protein samples enriched for PrP^{Sc} using ICAT and mass spectrometry

To assess whether we can identify PrP^{27-30} by LC-MS/MS in more complex protein mixtures, we performed a quantitative ICAT analysis by comparing brain homogenates from RML 5 inoculated terminally sick C57BL/6 or *tga20* mice with equally processed brain homogenates from mock inoculated control animals (C57BL/6 or *tga20* mice) using the established MPS labeling protocol. Prior to MPS treatment and ICAT labeling, protein extracts were enriched for PrP^{Sc} by differential precipitation with sodium phosphotungstic acid (NaPTA) and subsequently digested with PK. The protease was then heat-inactivated and the efficiency of the PK digest was determined by an anti-PrP Western blot. The PK resistant proteins were solubilized in 2% MPS by incubating the samples for 1 hour at 95°C. The RML 5 and mock protein samples were differentially ICAT labeled (i.e. mock inoculated: light; terminally sick: heavy), and purified as described. The purified peptides were separated over a reverse phase column prior to LC-MS/MS analysis. PrP^{Sc} could be detected in this analysis as judged by an increased abundance of the heavy labeled peptide (Fig. 35). A detailed analysis of the LC-chromatogram revealed that the light isoform of the cystein-containing peptide was not present in this sample. Additional proteins (total of 26) were detected, however all showing an unaltered isotope ratio. The only protein that displayed differentially in the two samples was the internal positive control PrP^{Sc}.

We have repeated this type of experiments and were able to detect PrP^{Sc} in as little as 100µg of PK (no NaPTA precipitation performed) treated 10% brain homogenate of terminally sick C57Bl/6 mouse brains (data not shown).

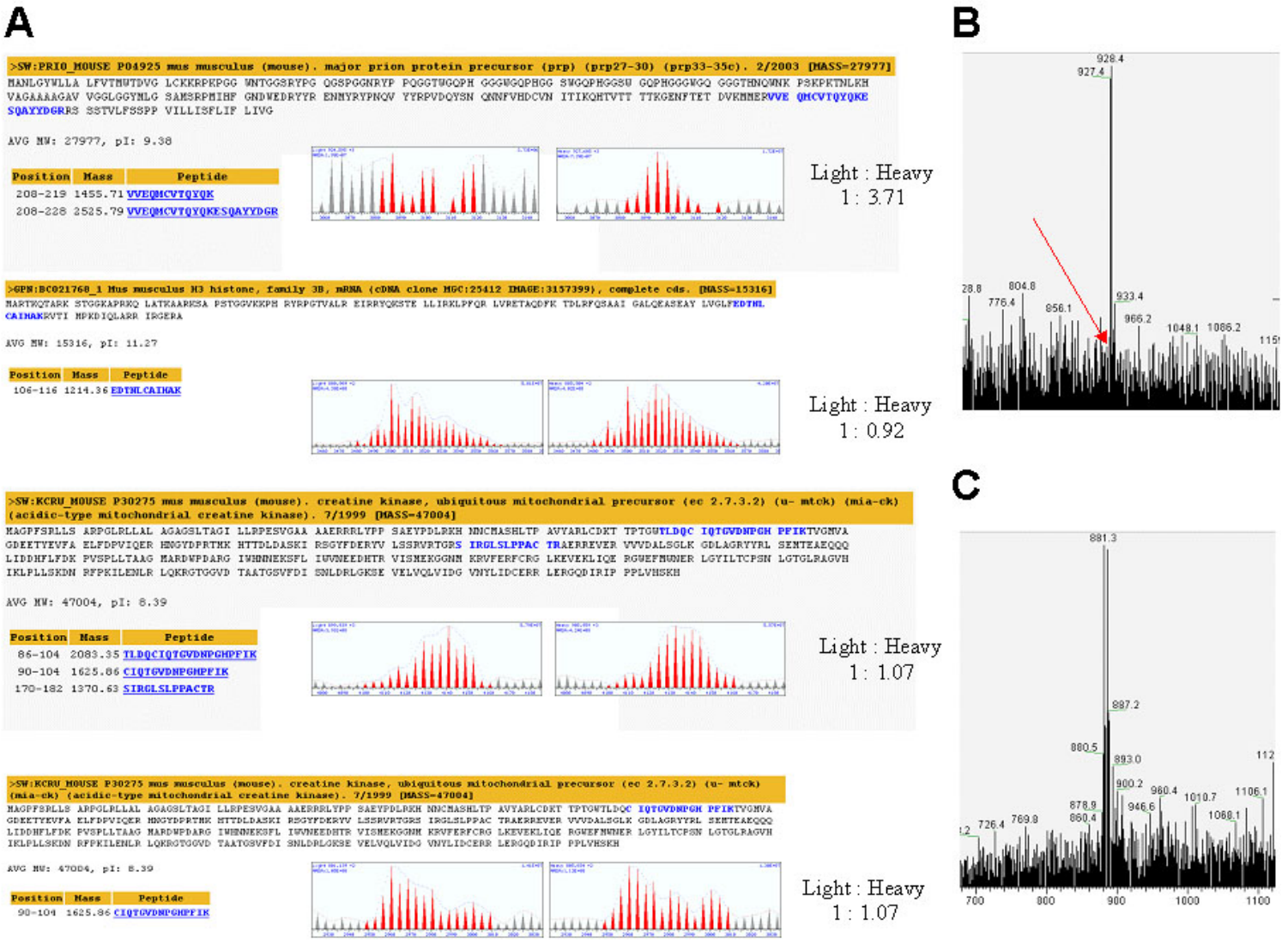


Figure 35. A MS-based elution profile for PrP and three representative peptides. Protein samples from mock or RML5 inoculated *tga*20 mice were NaPTA precipitated and PK-digested and subsequently ICAT labelled. Note the precise co-elution of light and heavy peptides and the conserved 1:1 ratio for the controls as compared to the altered ratio for PrP peptide. **B** Precursor peak for the heavy labelled PrP peptide. The corresponding light labelled peak is absent (red arrow). **C** Precursor peaks for the light and heavy labelled histone 3 peptide mentioned in A. As expected the precursor co-elute and appear with the same abundance.

DISCUSSION

Proteome analysis is gradually being applied to human brain samples from patients with neurodegenerative diseases or to the brains of transgenic mice that model human neurodegenerative diseases. Global profiles of protein expression are established and comparative proteome analysis is then performed allowing for identification of proteins differentially expressed in physiological and diseased states. Proteomics has also been applied to study specific proteins, the function of which may play a role in the pathological mechanism of neurodegenerative disease. Lately, several studies have been applying the techniques of electrophoresis, coupled with mass spectrometry, to specific enzymes in order to identify their role, their mechanism of action and their relationship with other proteins in the brain (Brining et al., 1999; Gibb et al., 2000; Lund et al., 2001). Several neurodegenerative diseases (*e.g.* Alzheimer's, Prion, and Huntington's disease) are characterized by the accumulation of protein aggregates. The proteins of these diseases lack any significant primary sequence homology, yet their aggregates possess very similar features, specifically, high beta sheet content, fibrillar morphology, protease resistance, and relative insolubility. This insolubility, however, hampers a more thorough analysis of these aggregates, in order to obtain an answer to the impact of these aggregates on neurons (neurotoxicity?) or the association with other proteins that might be involved in initiating the precipitation of these protein aggregates. The agents used to solubilize these protein aggregates (*e.g.* formic acid, urea, NaOH) are often not compatible with subsequent analysis by MS, since they hinder enzymatic digestion to obtain peptides of the aggregates. In this study, we have evaluated a new agent, referred to as MPS, for its solubilization efficiency of PrP^{Sc} compared to other known agents, that have been shown to disaggregate PrP^{Sc}, and for its compatibility with MS analysis.

High molar concentrations of urea and guanidinium have been shown to solubilize at least partially PrP^{Sc} (Safar et al., 1993). Therefore, we tested these agents for their usability in MS analysis. However, beside their limited solubilization efficiency, they had to be diluted or eliminated by dialysis prior to tryptic digestion, that represent time consuming steps and a risk to loose material. Moreover, diluting out detergents risk to propagate re-aggregation of PrP^{Sc} (Post et al., 1998). The new agent MPS, a mild denaturant agent, that renders proteins accessible to enzymatic cleavage, has been shown to solubilize PrP^{Sc} at least as good as GdnSCN and urea, and moreover

renders PrP^{Sc} PK sensitive. As denaturation of PrP^{Sc} has been consistently accompanied by a reduction in scrapie infectivity (Diener et al., 1982; Prusiner et al., 1993), we tested MPS for its inactivation activity. Incubation of RML 5 in 2% MPS at 95°C reduced infectivity by a factor of 5-6 logs LD50. However, it is questionable if MPS is the only factor that contributes to this reduction in infectivity, considering the results of incubation of RML 5 for 12h at 95°C. Independently of the presence of any detergent, PK or trypsin, proteins were lost after an incubation of RML 5 in H_20 for 12h at 95°C. In order to test the impact of heating on the loss of infectivity, an identical bioassay should be performed using H_2O in place of MPS. Additionally, samples that were treated with SDS also displayed a reduction of 3 logs LD50. This reduction is most likely due to enhanced PK activity in the presence of SDS, since it has been reported in several publications that only high concentrations of SDS significantly reduced the infectivity titer (Prusiner et al., 1993; Taylor et al., 1999). Moreover, it has been shown that the scrapie agent can be inactivated by prolonged protease K activity (Neary et al., 1991).

Furthermore, we have shown that the agent allows the detection of PrP^{Sc} tryptic peptides by MS. We found coverage of 60 % of the amino acid sequence of the PK resistant core of PrP^{Sc}. The possible sequence coverage is 83,5%, since the C-terminal fragment is expected to contain the GPI anchor. 23.5% of coverage of the amino acid sequence was missing because of the size of some peptides that is too small to be seen by the applied type of MS-analysis. Additionally, the new agent is compatible with the ICAT labeling and subsequent tryptic digestion to obtain the cystein containing and thus labeled peptides. Labeling in MPS was at least as good as labeling in urea. Quantitative analysis of protein samples enriched for PrP^{Sc} using ICAT and mass spectrometry revealed that PrP^{Sc} could be detected differentially in the infected versus non infected sample. The amino acid sequence of the prion protein contains two cysteins that can be labeled by the ICAT. However, we found only one cystein containing peptide. The other cystein containing peptide (amino acids 157-184) was probably not identified because of highly branched glycosyl groups linked to aspargin residue 181. Twenty-six additional proteins were detected all showing unaltered isotope ratio. However, we have not yet utilized the full potential of the method. The twenty-six additional proteins most likely represent the most abundant proteins in a PK-digested NaPTA precipitate. The small number of identifications in repeated analysis of the same preparation is due to the fact that we did not remove PK-cleavage

products and thus the sample complexity might still be relatively high. Furthermore, we have not further sub-fractionated the tryptic peptides by strong cation exchange (SCX) chromatography, which would again allow for the detection of proteins of lower abundance. Considering the difficulties in fully reconstituting continuous PrP conversion reactions with the purified PrP isoforms, it is tempting to conclude that there may be important conversion co-factors that remain poorly understood (DebBurman et al., 1997; Horiuchi and Caughey, 1999a; Saborio et al., 1999). Therefore, the improvement of detection and identification of low abundant proteins in PrP^{Sc} containing samples is crucial, since it might allow identifying such co-factors important for conversion and infectivity of the prion protein. In the case of the prion-infected material, however, sample processing and sub-fractionation of the material has to be done in contained laboratories in which some of these expensive platforms may not be available. Unfortunately, the inactivation of high titers of prion infectivity by the MPS procedure is not complete and thus sample handling cannot be accomplished outside the biosafety facilities.

Prion diseases are usually diagnosed clinically and confirmed by post-mortem histopathological examination of brain tissue. Presently, the most widely used diagnostic tests exploit the relative protease resistance of PrP^{Sc} in brain samples to discriminate between PrP^{C} and PrP^{Sc}, in combination with anti-PrP mediated detection of the proteinase K-resistant part of PrP^{Sc} (PrP^{27-30}) by Western blot or ELISA. However, no diagnostic test exists for the detection of prion diseases in live animals or humans. Due to the slow kinetics of accumulation of PrP^{Sc} and consequent low abundance of PrP^{Sc} in the pre-clinical stage of disease, the detection of the disease early in the incubation period is rendered very difficult. Mass spectrometry analysis allows femto- to attomole sensitivity and a high mass accuracy (Figeys et al., 1996; Valaskovic et al., 1996; Vorm and Roepstorff, 1994), thus ideal to detect low abundant PrP^{Sc}. Our established protocol using MPS could therefore be used for detection of PrP^{Sc} preferentially in easy accessible material such as urine and blood. Moreover, the ICAT labeling of PrP^{Sc} will allow for absolute quantification in combination with an internal standard. MPS enables a single buffer assay crucial for quantification, since it avoids excessive handling of samples and therefore loss of sample.

Another possibility of early diagnostics includes the finding of surrogate markers other than PrP^{Sc}. Comparative proteomic is widely used to detect differences in the

expression pattern of proteins between healthy and diseased organisms (Langen et al., 1999; Stoeckli et al., 2001). Such protein profiling might be helpful in identifying surrogate markers in blood or urine in the case of prion diseases. Our protocol might as well be useful for the application in finding surrogate markers in urine and blood using proteomics in prion diseased individuals.

METHODS AND MATERIALS

Construction and production of lentiviral vectors

Construction – The construction of the lentiviral vectors encoding PrP, ΔPrP and Dpl was performed using pCCLsin.PPT.CMV.EGFP.Wpre. The cDNA of PrP, ΔPrP and Dpl were obtained by PCR using primers containing the restriction sites BamHI and SalI (PrP- and ΔPrP - Primers: 5'-CGC GGA TCC AAT TTA GGA GAG CCA AGC AGA-3'; 5'- ACG CGT CGA CCA CGA GAA TGC GAA GGA ACA-3.Dpl-Primers: 5'- ACG CGGT CGA CAT TGA TCT TTA CTTT CAC AAT GAA – 3'; 5'- CGC GGA TCC ATT CAC CAT GAA GAA CCG GCT-3'). The transfer construct was digested with BamHI and SalI and the different cDNAs were ligated into the transfer construct and fused to the IRES.eGFP.

Production - The lentivirus vector was produced by co-transfection of human kidney 293T cells on 15cm plates with 30μg of transfer construct (gift from Prof. L. Naldini), 22.5μg of packaging plasmid (pCMVΔ8.91), and 5μg of envelop plasmid (pMD.G) (kindly provided by Prof D. Trono, University of Geneva Medical School, Geneva, Switzerland) by calcium- phosphate precipitation. After 14-16 hours, the medium was replaced with fresh media. After 48h respectively 72h, the conditioned medium was harvested, low-speed centrifuged, and filtered through a 0.45μm-pore-size filter. The viral vector stock was then concentrated by ultracentrifugation at 50000g for 1.5h. After the ultracentrifugation, the pellet was resuspended in sterile PBS and stored at 4°C. After the second collection of media and subsequent ultracentrifugation, the viral vector stocks were pooled and subjected to a second ultracentrifugation. The final pellet was suspended in sterile PBS, and aliquoted and stored at –80°C. An aliquot was used for p24 antigen detection by an enzyme-linked immunoabsorbent assay kit. Another aliquot was used for the titration of the viral vector stock. T293 cells were transduced with serially diluted supernatants.

Histopathology

Organs were fixed in 4% paraformaldehyde in PBS (pH 7.5), paraffin-embedded, and cut into 2μm sections. Brain sections were stained with hematoxylin-eosin, Luxol-

Nissl, and with commercial antibodies to phosphorylated neurofilament (Sigma). Antibody stainings were visualized using a peroxidase-anit-peroxidase method.

ICAT labeling

Bovine serum albumin (BSA; Sigma) or NaPTA precipitated samples were labeled either in urea (8M urea, 0.125% SDS, 50mM Tris-HCl pH 8.3, 0.5M EDTA) or in RapiGest labeling buffer (2% RapiGest SF, Waters; 50mM ammonium bicarbonate, 50mM Tris-HCl pH 8.3, 0.5M EDTA). Prior to the labeling, NaPTA precipitated samples were digested with 50µg/ml PK in the presence of 0.1% SDS for 30min at 37°C and thereafter heated at 95°C for 10min to inactivate PK. Additionally, prior to the labeling, proteins were reduced with 10mM TBP at 37°C for 30min. The labeling reaction was performed with either light or heavy label for 2h at RT and quenched with 10mM DTT. Light labeled and heavy labeled samples were combined and digested with trypsin O/N at 37°C. In the case of labeling in urea, the sample was diluted to a final concentration of 1M urea.

Immunocytochemistry

Cells on coverslips were fixed in 4% paraformaldehyde, blocked with 10% fetal calf serum, permeabilized if required with 0.1% Tween 20 in PBS containing 1% BSA, and incubated with primary antibody in blocking solution (1% BSA/PBS) for 1h at RT or O/N at 4°C. Subsequently to rinsing with PBS, cells were incubated with secondary antibody (anti-rabbit Alexa 546 or anti-mouse Alexa 546) in blocking solution for 1h at RT. Prior to mounting, cells were stained with Dapi. Cells were analyzed by fluorescent light microscopy (AxioM200, Zeiss) or confocal microscopy (Leica).

Immunoprecipitation assays

Myelin, spinal cord and cells were lysed in 0.5% Triton-X 100, 150mM NaCl, 50mM Tris-HCl pH 7.4, 5mM EDTA and protease inhibitors. The lysates were incubated with anti-PrP (6H4) antibody coated paramagnetic beads (Dynal) for 1.5h at RT. The beads were washed twice with lysis buffer and once with PBS. The proteins were dissociated from the beads by addition of SDS sample buffer and boiling for 5min,

prior to being separated on a 4-20% SDS-PAGE (Novex precast gel, Invitrogen) and transferred to nitrocellulose membrane.

Infectivity bioassay

To test if, and to what extent, enhanced Proteinase K (Sigma) activity can reduce infectivity, 50 µl infected murine brain homogenate (10% RML 5) were added to 37µl of 0.2% SDS (final concentration: 0.09%). Of these 87µl, 10µl were diluted 10'000x (by sequential dilution). After the dilution, 4 *tg*a20 mice were inoculated intracerebrally (30µl/mouse). The presence of PrP^{Sc} in the sample was checked on a Western blot prior to inoculation. The remaining 76µl were further processed by digestion with 4µl of Proteinase K (PK) (1mg/ml; final concentration is 50µg/ml) at 37°C for 30min. The Proteinase K was then inactivated by incubating the sample at 95°C for 30min.. Complete digestion of PrP^{Sc} in the sample to PrP^{27-30} was tested by Western blot. 10µl of the resulting sample were diluted 20x into 190µl PBS, and 4 *tga*20 mice were inoculated i.c. (30µl/mouse).

To test if, and to what extent, the presence of 0.09% SDS (Sigma) can reduce infectivity, 37µl of 0.2% SDS and 4µl of PK (1mg/ml) were pooled and incubated at 37°C for 30min followed by an incubation at 95°C for 30min to inactivate PK. The sample was then spiked with 50 µl of 10% RML 5. 10µl of the sample were diluted 10'000x (by sequential dilution). 4 *tga20* mice were inoculated intracerebrally (30µl/mouse). Also here, the presence of PrP^{Sc} is checked on a western blot. To the remaining 80µl, an equal amount of 4% RapiGest was added (final concentration is 2% RapiGest) and incubated for 60min at 95°C. Subsequently, 10µl of the sample were 20x diluted and 4 *tga*20 mice inoculated (30µl/mouse). The effect of RapiGest on PrP^{Sc} in the presence of inactivated PK and SDS is tested on a western blot prior to inoculation. Incubation time until development of terminal scrapie sickness was determined, and total infectivity titers were calculated using the relationship y=11.45-0.088x, where y is the LD_{50} and x is the incubation time (days) to terminal disease.

Isolation of myelin

Myelin from mouse brain was purified essentially as described by Norton and Poduslo (1973). In brief, tissue homogenates of 5% (w/v) were prepared from brain in 0.32M

sucrose. Some of the homogenized brain was stored at –80°C. The rest was layered over 0.85M sucrose, centrifuged at 75000g for 30min. The crude myelin was collected from the interface, suspended in water and centrifuged at 75000g for 15min. The resultant pellet was osmotically shocked in deionized water to remove contaminants from within myelin vesicles, and centrifuged twice at 12000g for 15min. The pellets were suspended in 0.32M sucrose, layered over 0.85M sucrose, and centrifuged at 75000g for 30min. The myelin was again collected from the interface, resuspended in deionised water and pelleted at 75000g for 15min to remove residual sucrose. The myelin was resuspended in H2O and stored at –80°C.

Primary cell cultures

Cerebellar granule cells were isolated from 7-days-old mice as describe previously (Schousboe et al., 1989). Neurons were plated onto 100μg /ml (250 μg /ml of for glass surfaces) poly-L-lysine coated dishes at a density of 1 x 10^6 cells/ml and cultured in Eagle's basal medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 25mM KCl, 2mM L-glutamine, 100 units/ml penicillin, and 100μg/ml streptomycin. To prevent growth of glial cells, cytosine arabinoside (10μM) was added to the cultures 24 hours after seeding. Glial fibrillary acid protein-positive cells were < 5%.

Mixed glial cell cultures containing oligodendrocytes and astrocytes were produced from 1-day-old neonatal mice as describe previously (Trotter et al 1989, McCarthy and de Vellis, 1980). Cultures were prepared with high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and replenished, on day 4 and every 3-4 days thereafter for 10 days, with DMEM plus 10% heat-inactivated horse serum. Oligodendrocytes were purified from mixed glial cultures by differential detachment, and negative selection of microglia by adherence to hydrophobic plastic. Purified oligodendrocytes were then plated onto either a glass or plastic culture chambers coated with 100μg/ml and 10μg/ml poly-L-lysine, respectively. Oligodendrocyte precursor cells were expanded with PDGF and FGF supplemented SATO medium for 2 days and subsequently differentiated with 1% horse serum supplemented SATO medium. After 3 days of differentiation, cells were washed with PBS and homogenized in lysis buffer (0.5% NP40, 0.5% sodium deoxycholate, protease inhibitors (Roche Molecular Biochemicals) in PBS).

Homogenate was then incubated on ice for 30min and centrifuged at 10000rpm for 15min at 4°C. Equal amounts of lysate were then processed for immunoblotting.

Raft preparation

50 µg of myelin membrane preparation was added to 1.5 ml 20 mM CHAPS or 1% Triton X-100 in TNE (25 mM Tris-HCL pH 7.8, 150 mM NaCl, 5 mM EDTA) containing protease inhibitors (Complete, Mini; Roche) and incubated on ice for 30 minutes. 3 ml OptiPrep^{TM} (Axis Shield, Oslo, Norway) was added giving a final concentration of 40% OptiPrep and 30% OptiPrep (10 ml) and 5% OptiPrep (2.3 ml) was carefully overlayed. The discontinues gradients were centrifuged for 14 h at 23.400 rpm (4°C) in a Ti 32.1 rotor (Beckman) and 11 fractions of 1.5 ml was collected from the top.

Semi-thin sections and electron microscopy

Mice were perfused with ice cold 4% PFA/3% glutaraldehyde, brain and spinal cord were removed and immersed in the same solutions and kept at 4° until processing. Tissues were embedded in Epon, semi-thin sections were cut and stained with toluidine blue or used for electron microscopy.

Sodium phosphotungstic acid precipitation (NaPTA)

NaPTA precipitation was performed as described (Safar et al., 1998; Wadsworth et al., 2001). In brief, 10% (w/v) homogenates from infected or non-infected murine brains were prepared in 0.32M sucrose. Gross cellular debris were removed by centrifugation at 1000rpm for 1 min in a microfuge (Eppendorf). 500µl of the resultant supernatant was mixed 1:1 (v/v) with 4% (w/v) sarkosyl (laurylsarcosine sodium salt; Sigma) prepared in PBS pH 7.4 and incubated for 15min at 37°C with constant agitation. Samples were adjusted to final concentrations of 50units/ml benzonase (benzon nuclease; Merck) and 1mM $MgCl_2$ and incubated for 30min at 37°C with constant agitation. Subsequently, samples were adjusted with 81.3µl of pre-warmed and less than two-week-old NaPTA-stock solution (4% NaPTA/170mM $MgCl_2$, pH 7.4, prepared in H20 and titrated to pH 7.4 with H20). Samples were incubated at 37°C for 30min with constant agitation prior to centrifugation at 14000rpm (15’800xg) for 30min in a microfuge prewarmed at 37°C (Eppendorf).

After careful isolation of the supernatant, the pellet was resuspended to 20µl final volume with PBS containing 0.1% (w/v) sarkosyl pH 7.4. Thereafter, resuspended pellet was PK digested (50µg/ml) at 37°C for 45min and heat inactivated for 30min at 95°C. RapiGest (Waters) was added to the sample at a final concentration of 2%. The sample was then incubated at 95°C for 1h and was further processed by tryptic digestion or ICAT labeled followed by tryptic digestion at 37°C O/N. Sample was then purified over SEPAK column and speed-vacuum dried. RapiGest was finally removed by acidic cleavage and the sample was analyzed using LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA).

Titration of virus

150000 T293 cells/ well in a 24 well-plate were seeded in 500µl of DMEM and 10% FCS. Serial dilutions of concentrated virus stock were prepared in a 96 well plate: 5µl of concentrated virus were added to 200µl PBS and mixed thoroughly. 50 µl from this dilution were diluted in the next well and consequently diluted in 6 further wells (dilution factor 300000). Of each dilution 10µl were added to the seeded T293 cells. After 48h, cells were trypsinized and fixed in 4% paraformaldehyde for at least 20min. The fixed cells were transferred to FACS tubes and analyzed for eGFP positive cells by FACS on a four-color FACSCalibur flow cytometry (Becton Dickinson). Titers were determined according to following formula: (%GFP positive cells x dilution factor x number of cells in assay)/1000.

Western blot

Lysates were run on SDS-PAGE (4-20% precast gel, Novex) and blotted on nitrocellulose membranes (Schleicher & Schuell, Dassel). Membranes were blocked with 5% (w/v) Top Bloc (Juro AG, Luzern) in TBS-T at RT for 1 hr, incubated with 20ml TBS-T/1% Top Bloc containing the respective antibody at RT under agitation for 1 hr, washed 3x10’ in TBS-T, and incubated with 20ml TBS-T/1% Top Bloc and secondary antibody at RT under agitation for 1 hr. Membranes were washed 3x15’ in TBS-T, and developed using ECL detection reagents. Signals were recorded on film and/or quantified using a Kodak Image Station.

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CURRICULUM VITAE

First Name: **Nathalie**

Last Name: **Braun**

Date of Birth: 04.05.1972

Place of Birth: Kreuzlingen TG

Nationality: Swiss (Kreuzlingen TG)

EDUCATION

2000 – Present

MD-PhD at the Institute of Neuropathology, University of Zurich, Prof. A. Aguzzi

- PhD thesis: "Biochemical and molecular studies of the prion protein"
- MD thesis: "Evaluation of plasminogen affinity assay for the isolation of a PrP^{Sc} protein complex: association and dissociation of the infectious prion protein and plasminogen."

1999 – 2001

Undergraduate studies in biology for MD-PhD

- Studies in biochemistry, University of Zurich
- Studies in molecular and cellular biology, University of Lausanne

1993 - 1999

Studies in Medicine, University of Bern and Lausanne

- 1999 Final medical exam, second part, University of Lausanne
- 1997 – 1998 Internship: 3 months at the Albert Schweitzer Hospital, Lambarene, Gabon and 1 month at the Mount Sinai Hospital, Toronto, Canada
- 1993 - 1995 Premedical studies, University of Bern

PUBLICATIONS

Polymenidou, M., Heppner, F.L., Pellicioli, E.C., Urich, E., Miele, G., **Braun, N.**, Wopfner, F., Schatzl, H.M., Becher, B., Aguzzi, A. (2004), Humoral immune response to native eukaryotic prion protein correlates with anti-prion protection. *Proc Natl Acad Sci U S A.* 1-7

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* Equal contribution